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(54) Title: P53-MEDIATED APOPTOSIS

(57) Abstract

The invention is directed to methods of reducing the viability of a proliferating mammalian cells such as cancer cells. In one method cells deficient in p53 activity and in p53 suppressor activity of one or more p53-interacting regulatory proteins cell viability is reduced by increasing the level or activity of p53 in the cell. In another method viability of cells exhibiting p53 activity and p53 suppressor activity of one or more p53-interacting regulatory proteins is reduced by reducing the suppressor activity of the one or more p53-interacting regulatory proteins. Further, cell viability is reduced in cells deficient in p53 activity and exhibiting p53 suppressor activity of one or more p53-interacting regulatory proteins by a method that includes: (a) increasing the level or activity of p53 in the cell, and (b) reducing the suppressor activity of the one or more p53-interacting regulatory proteins. Also, included are methods of selectively reducing the viability of proliferating cancer cells compared to nonproliferating normal cells within a mixed population of cells and to methods of selectively reducing the viability of chronic granulocytic leukemia cells within a sample of proliferating bone marrow cells.

P53-MEDIATED APOPTOSIS.

Throughout this application various publications are referenced within parentheses. The
5 disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

10   This invention relates generally to the regulation of cell growth and differentiation and, more particularly, to the modulation of cell cycle regulatory proteins for the therapeutic treatment of uncontrolled cell growth.

15   It is becoming increasingly apparent that carcinogenesis is the result of the deregulation of the intricate control mechanisms which have evolved in multicellular organisms to control normal cell growth and differentiation. The protein products of oncogenes,
20 first identified as the acute transforming genes transduced by retroviruses, are a group of dominantly acting genes, including growth factors and their receptors, as well as second messengers and mitogenic nuclear proteins activated by growth factors, involved in
25 cell division. The molecular cloning and characterization of the tumor suppressor genes p53 and Rb, or retinoblastoma, shows that both of these proteins suppress cell growth.

30   It has recently become apparent that normal hematopoiesis is dependent not only on pathways which positively or negatively regulate cell growth, but also on factors which regulate apoptosis, or programmed cell death. Growth factors are necessary for both lymphoid
35 and myeloid progenitor cell survival, and certain

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cellular and viral transforming genes function to prevent
apoptosis.

The standard methods of treatment for cancer
currently include surgery, radiation therapy, and
5 chemotherapy using cytotoxic drugs. However, for therapy
to be effective, essentially all cancer cells need to be
eradicated. Even if a very small fraction of the
original population remains, over time, the cancer can
reappear. An additional problem in treating cancer
10 results from metastasis. Thus, removal of a primary
tumor is only effective if done before any metastasis has
occurred. High dose chemotherapy +/- radiation therapy
followed by bone marrow transplantation is one approach
to the treatment of metastatic cancer. However, certain
15 cancers in which high dose chemotherapy might be useful,
i.e., small cell cancer of the lung and breast cancer,
commonly metastasize to the bone marrow. Thus, further
techniques to rid the bone marrow samples of tumor cells
are needed so tumor cells are not reinfused with the bone
20 marrow.

In an effort to improve cancer therapy,
treatments have now included the use of gene therapy to
inhibit cellular growth. Expression of regulatory
proteins such as p53 and Rb in animal models has been
25 shown to inhibit proliferation of cancer cells and to
modestly prolong the animals life. However, such
treatments require long-term expression in essentially
every abnormal cell. Current methods cannot achieve this
requirement. Moreover, expression of these regulatory
30 proteins has only been shown to inhibit proliferation,
and not to effect cell viability as would be preferred
for the most effective form of treatment.

Thus, there exists a long-felt need for a more
efficient and less toxic means of cancer treatment which

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results in the loss of cell viability. The present
invention satisfies these needs and provides related
advantages as well.

SUMMARY OF ·THE INVENTION

5   The invention is directed to a methods of
reducing the viability of a proliferating mammalian cells
such as cancer cells. In one method cells deficient in
p53 activity and in p53 suppressor activity of one or
more p53-interacting regulatory proteins cell viability
10   is reduced by increasing the level or activity of p53 in
the cell. In another method viability of cells
exhibiting p53 activity and p53 suppressor activity of
one or more p53-interacting regulatory proteins is
reduced by reducing the suppressor activity of the one or
15   more p53-interacting regulatory proteins. Further, cell
viability is reduced in cells deficient in p53 activity
and exhibiting p53 suppressor activity of one or more
p53-interacting regulatory proteins by a method that
includes: (a) increasing the level or activity of p53 in
20   the cell, and (b) reducing the suppressor activity of the
one or more p53-interacting regulatory proteins. Also,
included are methods of selectively reducing the
viability of proliferating cancer cells compared to
non-proliferating normal cells within a mixed population
25   of cells and to methods of selectively reducing the
viability of chronic granulocytic leukemia cells within a
sample of proliferating bone marrow cells.

BRIEF DESCRIPTION OF THE DRAWINGS

30   Figure 1 shows RNA and protein analysis of
p53^{ts}neo transfectants.

             Figure 2 shows the loss of viability induced by
wild-type p53.

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Figure 3 shows DNA fragmentation in p53 induced apoptosis.

Figure 4 shows cell cycle analysis by quantitative flow cytometry of clone C9.

5   Figure 5 shows loss of viability induced by wild-type p53.

Figure 6 shows cell cycle analysis by quantitative flow cytometry.

Figure 7 shows DNA fragmentation correlates
10   with commitment to cell death.

Figure 8 shows viability and differentiation of DP16-1 MEL cells grown in media containing 1.6% DMSO.

Figure 9 shows DMSO induces translocation of p53^{ts} into the nucleus and subsequent apoptosis of p53^{ts}neo
15   transfected DP16-1 cells.

Figure 10 shows analysis of cells for expression of the exogenous p53 and c-myb transcripts.

Figure 11 shows viability and differentiation of c-myb transfected cells grown in media containing
20   DMSO.

Figure 12 shows the coimmunoprecipitation of c-myb and p53.

Figure 13 shows Expression of c-myc in p53^{ts}neo transfected DP16-1 cells.

25   Figure 14 shows the analysis of exogenous c-myc mRNA and p53 mRNA.

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Figure 15 shows conformation of p53^{ts} in DMSO-treated DP16-1 cells.

Figure 16 shows the viability of DP16-1 cells transfected with c-myc with or without mutant p53

5   Figure 17 shows the differentiation of DP16-1 cells transformed with p53 and c-myc during DMSO-induced differentiation.

DETAILED DESCRIPTION OF THE INVENTION

10   This invention is directed to simple and effective methods for controlling and preventing unwanted cell growth. The methods exploit the genetic differences between proliferating cells such as cancer, for example, and normal cells and utilize the functions of cell cycle
15   regulatory proteins to specifically kill only the proliferating cells. This genetic selectivity offers several advantages over conventual cancer therapies. For example, specific genetically based compounds can be used to modulate uncontrolled cell growth which are markedly
20   less toxic than conventual treatments and thus, eliminate or reduce damage to normal tissues. Moreover, the methods are amenable to the selective killing of cancer cells which are often resistant to conventual treatment. Finally, the novel methods described herein offer the
25   additional advantage of augmenting the efficacy of conventual therapy by combining the two approaches.

In one embodiment, proliferating tumor cells are specifically killed over non-proliferating normal cells by inducing apoptosis using the tumor suppressor
30   gene product p53. In proliferating tumor cells that are deficient in both p53 and other cell cycle regulatory proteins that can modulate the apoptotic function of p53, specific killing is accomplished by directly expressing a

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functional p53 protein. An adenovirus-derived vector is
used to deliver and express a functional p53 encoding
nucleic acid by infection of cells in the proliferating
area. Since apoptotic induction is rapid, p53 expression
5   can be accomplished by either transient or stable
expression. Viability of normal, non-proliferative cells
expressing p53 will unchanged, but, proliferating tumor
cells will be arrested in G1 of the cell cycle and under
go apoptotic cell death.

10   In another embodiment, proliferating tumor
cells that express functional p53 and also express the
above mentioned p53-interacting cell cycle regulatory
proteins are specifically killed by eliminating the
function of the interacting proteins. Inhibition of the
15 cell cycle regulatory proteins releases p53 from their
suppressing activity and allows p53-induced apoptosis to
occur in the proliferating cells. Function is eliminated
using antisense oligonucleotides to one or more of the
p53-interacting proteins. Alternatively, adenovirus-
20 mediated gene transfer can be employed to express
antisense nucleotide sequences within the proliferating
cells, or to introduce a dominant-negative form of the
p53-interacting protein.

          In yet another embodiment, proliferating tumor
25 cells deficient in p53 function but expressing p53-
interacting regulatory proteins are specifically killed
by first expressing p53 through adenovirus-mediated gene
transfer and then selectively inhibiting expression of
the p53-interacting cell cycle regulatory protein or
30 proteins. Inhibition is again accomplished by
administering antisense oligonucleotides or a dominant-
negative form of the p53-interacting protein to decrease
expression or activity of the p53-interacting proteins.

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In still a further embodiment, p53-induced apoptosis is used to purge chronic granulocytic leukemia (CML) from bone marrow samples that are to be used for autologous bone marrow transplants. As with the

5   selective killing of proliferating cells to control uncontrolled cell growth, manipulation of the expression of p53 and cell cycle regulatory proteins is used to selectively kill CML in low density mononuclear bon marrow cells isolated from a patient with CML. The bone

10   marrow cells are first treated with macrophage inflammatory factor (MIP-1α) to arrest normal hematopoietic cells in G0. Arrested cells are not susceptible to p53-induced apoptosis whereas, in the functional absence of p53-interacting cell cycle

15   regulatory proteins, proliferating cells are susceptible to p53-mediated cell death. Induction of p53-mediated apoptosis is accomplished by inhibiting the suppressor effects of such interacting cell cycle regulatory proteins by culturing the bone marrow cells in the

20   presence of c-myb antisense oligonucleotides. CML cells, which are unresponsive to MIP-1α, will continue to cycle and are committed to apoptotic cell death.

As used herein, the term "p53" or "p53 tumor suppressor gene product" refers to a polypeptide having a

25   molecular weight of 53,000 kD and is a nuclear regulatory protein. The p53 nucleotide and deduced amino acid sequences have been described for a broad range of species and are well known in the art. A description of such sequences can be found in, for example, Baker et

30   al., Science 244:217-221 (1989); Bischoff et al., Mol. Cell Biol. 12:1405-1411 (1992) and Chin et al., Science 255:459-462 (1992), all of which are herein incorporated by reference. Nucleic acids encoding a p53 tumor suppressor gene product are available within the art or

35   can be obtained by isolating a cDNA using the published sequences and standard methods within the art. It is

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understood that limited modifications may be made without
destroying the biological activity of p53, and that only
a portion of the entire primary structure may be required
in order to effect activity. These modifications may be
5   deliberate, as through site-directed mutagenesis, or may
be accidental such as through mutation in hosts which are
p53 producers. All of these modifications are included
as long as p53 activity is retained. Further, various
molecules can be attached to p53, for example, other
10   proteins, carbohydrates, or lipids. Such modifications
are included within the definition of p53.

As used herein, the term "p53 activity" refers
to the biological functions of the p53 tumor suppressor
gene product. It is understood that proteins exhibiting
15   similar biological function but having different
nucleotide and amino acid sequences than p53 or a p53
homolog, are also intended to fall within the definition
of proteins, or cells expressing proteins having p53
activity. The biological functions of p53 include, for
20   example, the suppression of cell growth, such as that
described by Friend et al., Nature 323: 643-646 (1986),
and the induction of programmed cell death or apoptosis.
Induction of cell death through p53 is specific to
proliferating cells and can be suppressed by cell cycle
25   regulatory proteins. Thus, the term "p53-mediated
apoptosis" as used herein refers to the commitment of
proliferating cells to programmed cell death that is
caused by the presence of p53 activity.

As used herein, the term "p53 suppressor
30   activity" refers to activity mediated by, for example, a
protein that is capable of reducing or inhibiting one or
more biological functions of p53. Examples of proteins
exhibiting p53 suppressor activity include cell cycle
regulatory proteins that can bind p53 and prevent
35   apoptosis. Such proteins can include, for example, c-

myb, c-myc and bcl2. Thus, the use of the term "p53-
interacting regulatory proteins" is intended to refer to
those cell cycle regulatory proteins, for example, that
reduce or inhibit the activity of p53 and p53-like

5   proteins. Modification of p53 activity can be by direct
interaction, such as by the physical binding of p53, or
can be indirect, such as by the functional modulation of
p53 activity through a p53 regulatory pathway.

As used herein, the term "expressing" refers to
10   the biosynthesis of a nucleic acid or polypeptide through
endogenous cellular mechanisms. The term includes all
necessary steps for the transcription and/or the
transcription and translation of a genetic sequence into
the nucleic acid or polypeptide. The genetic sequence
15   can be a natural sequence or be derived by recombinant or
by synthetic means. Also included are any regulation,
processing and sorting events required to achieve a
desired outcome, such as splicing of introns and post
translational modifications.

20   As used herein, the term "substantially" when
used in reference to nucleotide sequence complimentarity
refers to the degree to which two sequences are
complimentary so as to allow specific hybridization under
a given set of conditions. Hybridization principles and
25   methods for determining hybridization specificity are
well established and are known by one skilled in the art.
Such principles and methods can be found in, for example,
Sambrook et al., Molecular Cloning: A Laboratory Manual,
Cold Spring Harbor Laboratory, New York (1992), and in
30   Ausubel et al., Current Protocols in Molecular Biology,
John Wiley and Sons, Baltimore, MD (1989), both of which
are herein incorporated by reference. Thus, it is not
necessary that two nucleic acids exhibit sequence
identity to be substantially complimentary, only that
35   they can specifically hybridize or be made to

specifically hybridize without detectible cross
reactivity with other similar sequences.

As used herein, the term "effective amount"
refers to the amount of an agent or compound required to
5   obtain a desired outcome. For example, if a compound is
required to arrest proliferation, than the amount added
to the cells that produces this response is considered to
be an effective amount. One skilled in the art knows, or
can determine what an effective amount is for a
10 particular cell type or tissue, or, what constitutes an
effective amount for a therapeutic treatment.

As used herein, the term "biologic response
modifier" refers to a peptide, polypeptide or protein
that is capable of altering cellular growth and
15 differentiation functions. Such molecules can be of
natural or of synthetic origin so long as they are
capable of changing a particular growth or
differentiation function in a predetermined way.
Examples of biologic response modifiers include growth
20 factors and mitogens which are known to either inhibit or
promote these functions. One specific biologic response
modifier which is capable of arresting cell proliferation
is macrophage inflammatory factor (MIP-1α), for example.
Macromolecules and compounds other than peptides,
25 polypeptides and proteins are also intended to be
included within the definition. Such other
macromolecules and compounds can include, for example,
carbohydrates, lipids, amino acids and drugs such as
mimosine.

30   Cellular growth and differentiation is
controlled by numerous distinct as well as interacting
processes. The difference between normal and
uncontrolled cell growth and differentiation relies on
the accurate regulation of these processes. Numerous

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proteins and their encoding genes have been identified
that play a role in one or more growth and
differentiation processes. For example, tumor suppressor
gene products, such as p53 and Rb, suppress cell
5   proliferation, Friend et al., supra and Marshall, C.J.
Cell 64:313-326 (1991). Deficiencies in these gene
products can lead to uncontrolled cell growth, such as
cancer, for example. A specific example is in the case
of erythroleukemia and erythroleukemia cell lines where
10   there is generally a lack of expression, or the
expression of a mutant p53 tumor suppressor gene product.

The involvement of p53 in cellular control can
be complex and depend on the cell type and state of
differentiation. As stated above, myeloid leukemic cell
15   lines frequently fail to express p53, however, lymphoid
leukemic cell lines characteristically overexpress this
protein. Moreover, some lines that express normal, or
wild type, p53 such as ML 1, for example, lack expression
during logarithmic growth but show increased levels with
20   induced differentiation. Further, mouse erythroleukemia
cell lines (MEL) expressing mutant p53 typically show a
decrease in expression with induced differentiation. p53
also plays a role in the control of proliferation of
early myeloid precursors.

25   The results generated in the examples which
follow demonstrate that expression of p53 protein in
erythroleukemia cells is associated with growth arrest
and that this arrest occurs in actively cycling
populations of cells predominantly in the G0-G1
30   transition and G1 phase of the cell cycle. In addition,
cell death occurs rapidly following p53 induced growth
arrest in proliferating cells but not in non-cycling or
differentiated cells. The cell death induced by p53 is
accompanied by endonucleolytic cleavage of genomic DNA
35   characteristic of apoptosis. Importantly, p53 induced

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cell death is not solely a function of its ability to block cells in G1 indicating that p53 expression affects cell viability independent of growth arrest directly attributable to p53. Thus, p53 has two related but

5   separable roles as a cell cycle regulatory protein; the control of cell proliferation and the control of cell viability.

In addition to p53, other cell cycle regulatory proteins exist that can control growth and

10   differentiation processes. These cell cycle regulatory proteins include, for example, the nuclear proteins c-myb and c-myc and non-nuclear proteins such as bcl2. Also, members of the tyrosine kinase family of oncogenes can be included as cell cycle regulatory proteins. This family

15   includes, for example, src, ras, grb2, raf and bcr/abl. For the specific case of hematopoietic cells, expression of c-myb and c-myc are essential for entry into S-phase of the cell cycle and for DNA synthesis, (Yonish-Roach et al., Nature 352:345 (1991). In addition to their role in

20   cell cycle transit in normal cells, constitutive expression of c-myb also blocks MEL cell differentiation whereas expression of c-myc in arrested cells induces apoptosis. Numerous other gene products known within the art also exhibit cell cycle regulatory functions. One

25   example is the bcl2 gene product which functions to promote cell viability by blocking apoptosis.

As described here and below, there are several cell cycle regulatory proteins that are able to modify the p53-mediated apoptosis of proliferating cells. These

30   p53-interacting regulatory proteins, which include those described above, either alleviate or delay p53-induced cell death. For example, c-myb and c-myc both partially inhibit p53-mediated apoptosis whereas bcl2 delays this process. Additionally, mdm2 binds and inactivates p53 to

35   a significant extent.

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Conversely, just as p53-interacting regulatory proteins modify p53-mediated apoptosis, p53 can also modify the function of these regularly proteins. For example, the c-_myb_ dependent differentiation block in MEL

5   cells is overcome in the presence of p53 just as the c-_myc_ dependent apoptosis is inhibited in the presence of p53. Moreover, many tumor cells contain a mutant form of p53 that is unable to induce apoptosis, however, still retains its ability to disrupt myc-induced apoptosis of

10 arrested cells. Thus, the interaction of cell cycle regulatory proteins, whether tumor suppressor genes or oncogenes, is complex with each protein modifying the phenotypic effects of the other in unexpected ways.

The invention provides a method of reducing the

15 viability of a proliferating mammalian cell deficient in p53 activity and in p53 suppressor activity of one or more p53-interacting regulatory proteins. The method includes increasing the level or activity of p53 in the proliferating mammalian cell.

20   The invention also provides a method of reducing the viability of a proliferating mammalian cell exhibiting p53 activity and p53 suppressor activity of one or more p53-interacting regulatory proteins. The method includes reducing the suppressor activity of the

25 one or more p53-interacting regulatory proteins.

The invention further provides a method of reducing the viability of a proliferating mammalian cell deficient in p53 activity and exhibiting p53 suppressor activity of one or more p53-interacting regulatory

30 proteins. The method includes increasing the level or activity of p53 in the cell, and reducing the suppressor activity of the one or more p53-interacting regulatory proteins.

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The above described functions of cell cycle
regulatory proteins can be advantageously used to
regulate uncontrolled cell growth of proliferating
mammalian cells by inducing p53-mediated apoptosis in the
5 proliferating cell or population. Proliferating
mammalian cells susceptible to p53-mediated apoptosis
include, for example, cancer cells and other neoplasia,
particularly of the hematopoietic cell lineage. Cancer
cells other than of the hematopoietic lineage can
10 include, for example, cell types as diverse as lymphoma
cells, breast cancer cells, prostate cancer cells, oat
cell carcinoma cells, lung cancer cells, colon cancer
cells, bladder cancer cells, brain tumor cells, head and
neck cancer cells, and pancreatic cancer cells. Numerous
15 other cell types susceptible to p53-mediated apoptosis
exist as well and are known, or can be determined by one
skilled in the art. Thus the invention provides for a
method of reducing the viability of a proliferating
mammalian cell wherein the cell is any one of a variety
20 of cancer cell types.

The method by which cell viability can be
reduced will depend on the genetic background of the cell
as to whether they exhibit p53 activity and/or p53
suppressor activity and whether the cell is actively
25 proliferating. In the case where cells are deficient in
both of these activities, cell death can be induced by
increasing the level or activity of p53. Such increases
can be obtained by introducing into the cell an
expression vector containing a p53 encoding nucleic acid.
30 Expression of the p53 gene product in a proliferating
cell will lead to growth arrest in the G1 phase of the
cell cycle and result in apoptotic cell death. Non-
proliferating or arrested cells, such as normal
terminally differentiated cells, for example, will be
35 unaffected by the expression of p53.

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In the opposite case where the cells exhibit
both p53 activity and p53 suppressor activity, cell
viability can be reduced by inhibiting or decreasing the
suppressor activity. Inhibition of p53 suppressor
5   activity essentially produces cells exhibiting only p53
activity and the cells are therefore susceptible to p53-
mediated apoptosis. Again, p53 activity will only lead
to cell death in proliferating cells and not in arrested
cells.

10   The inhibition of p53 suppressor activity can
be obtained by a variety of methods. Such methods
include, for example, directly inhibiting suppressor
activity by treating the cells with compounds or agents
that bind to and inactivate molecules, such as p53-
15 interacting regulatory proteins, that exhibit the p53
suppressor activity. Another methodology includes
introduction into the cell of a dominant-negative mutant
of the p53-interacting regulatory proteins. Such mutants
inhibit the function of their wild-type counterparts. A
20 specific example of a dominant-negative mutant of a p53-
interacting regulatory protein is mbm2.

Alternatively, indirect inhibition of
suppressor activity can be obtained by using agents such
as growth factors, growth factor agonists or antagonists,
25 antibodies and the like that modulate the levels of the
p53-interacting proteins. Another method is to inhibit
the synthesis of such proteins through the use of
antisense or triplex oligonucleotides, analogues or
expression constructs. This method entails introducing
30 into the cell a nucleic acid sufficiently complementary
in sequence so as to specifically hybridize to the target
p53-interacting regulatory protein encoding gene or
message. Triplex inhibition relies on the
transcriptional inhibition of the target gene and can be
35 extremely efficient since only a few copies per cell are

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required to achieve complete inhibition. Antisense
methodology on the other hand inhibits the normal
processing, translation or half-life of the target
message. Such methods are well known to one skilled in
5   the art.

In the transitional case where cells are
deficient in p53 activity but exhibit p53 suppressor
activity, cell viability can be reduced by a combination
of increasing the level of p53 activity and also reducing
10 the level of p53 suppressor activity. Methods employed
to achieve these conditions are identical to those
described above. Again, the end result is the generation
of cells that exhibit essentially only p53 activity.

Recombinant methods known in the art can be
15 used to achieve any of the desired outcomes described
previously for the generation of a cell susceptible to
p53-mediated apoptosis. For example, vectors containing
p53 encoding or c-<u>myb</u>, c-<u>myc</u> or <u>bcl</u>2 antisense nucleic
acids can be employed to express protein or antisense
20 message and thereby elevate the level or activity of p53
or reduce the p53 suppressor activity. Such vectors are
known or can be constructed by those skilled in the art
and should contain all expression elements necessary to
achieve the transcription of antisense, or
25 transcription, translation, regulation, and sorting of
the protein. Other beneficial characteristics can also
be contained within the vectors such as mechanisms for
recovery of the nucleic acids in a different form.
Phagemids are a specific example of this because they can
30 be used either as plasmids or as bacteriophage vectors.
Examples of other vectors include viruses, such as
bacteriophages, baculoviruses and retroviruses, cosmids,
plasmids, liposomes and other recombination vectors. The
vectors can also contain elements for use in either
35 procaryotic or eucaryotic host systems. One of ordinary

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skill in the art will know which host systems are
compatible with a particular vector.

The vectors can be introduced into cultured
cells by any one of a variety of known methods within the
5   art. Such methods can be found described in Sambrook et
al., _Molecular Cloning: A Laboratory Manual_, Cold Spring
Harbor Laboratory, New York (1992), in Ausubel et al.,
Current Protocols in Molecular Biology, John Wiley and
Sons, Baltimore, MD (1989), and include, for example,
10   stable or transient transfection, lipofection,
electroporation and infection with recombinant viral
vectors. Introduction of nucleic acids by infection
offers several advantages over the other listed methods
which includes their use in both _in vitro_ and _in vivo_
15   settings. Higher efficiency can also be obtained due to
their infectious nature. Moreover, viruses are very
specialized and typically infect and propagate in
specific cell types. Thus, their natural specificity can
be used to target the vectors to specific cell types _in
20   vivo_ or within a tissue or mixed culture of cells. Viral
vectors can also be modified with specific receptors or
ligands to alter target specificity through receptor
mediated events.

A specific example of a viral vector for
25   introducing p53 encoding or other appropriate nucleic
acids for the induction of p53-mediated apoptosis is the
adenovirus derived vector Adenop53TK. This vector
expresses a herpes virus thymidine kinase (TK) gene for
either positive or negative selection and the p53 gene.
30   Alternatively, nucleic acids other, or in addition to the
p53 gene, can be placed in the vector to effect p53-
mediated apoptosis. Such other nucleic acids include
antisense sequences to p53-interacting regulatory
proteins. This vector can be used to infect cells that

have an adenovirus receptor which includes most cancers of epithelial origin.

The above described vector as well as others that exhibit similar desired functions can be used to
5   treat a mixed population of cells to selectively promote death of uncontrolled proliferating cells compared to normal cells. A mixed population of cells can include, for example, an _in vitro_ or _ex vivo_ culture of cells, a tissue or a human subject. Thus, the invention provides
10   a method of selectively reducing the viability of proliferating cancer cells compared to non-proliferating normal cells within a mixed population of cells. The method includes selectively inducing p53-mediated apoptosis in said proliferating cancer cells.

15   As with the methods described previously, the ability of p53 activity to specifically kill growing, but not resting cells can be used for the therapeutic treatment of cancer. In order to successfully treat a cancer using gene therapy by inducing p53-mediated
20   apoptosis, it is necessary to deliver the gene product to most or all of the target cancer cells. Also required is the essentially complete inhibition of p53 suppressor activity where appropriate by, for example, antisense oligonucleotides or expression. Thus, the genetic
25   background of the cancer cells will determine whether exogenous p53 activity and/or inhibition of p53 suppressor activity is required. Since p53 induction of cell death is rapid, requiring as little as a 4-6 hour exposure to p53 during the G1 phase of the cell cycle,
30   the expression of p53 need only be transient. Therefore, vectors that are efficient at infecting and transiently expressing a protein, such as the adenovirus vector above is advantageous for successful therapy.

Additional features can be added to the vector
to ensure its safety and/or enhance its therapeutic
efficacy. Such features include, for example, markers
that can be used to negatively select against cells
5   infected with the recombinant virus. An example of such
a negative selection marker is the TK gene described
above that confers sensitivity to the antibiotic
gancyclovir. Negative selection is therefore a means by
which infection can be controlled because it provides
10 inducible suicide through the addition of antibiotic.
Such protection ensures that if, for example, mutations
arise that produce mutant forms of p53, cellular
transformation will not occur. Moreover, features that
limit expression to particular cell types can also be
15 included. Such features include, for example, promoter
and expression elements that are specific for the desired
cell type.

A variety of modes can be used to administer
the vectors for the specific induction of p53-mediated
20 apoptosis within a diverse number of cancer cell types.
Types of caners include, for example, localized tumors as
well as diffuse soft tissue types. The mode of
administration will depend on the location and type of
cancer to be treated. For example, localized breast
25 cancers or head and neck cancers can be treated by
inoculation into they vascular system supplying the tumor
with nutrients. This mode as well as subcutaneous
injection can also be used to treat other types of
cancers wherein the procedure takes advantage of vector
30 target specificity. Alternatively, local administration
by, for example, direct inoculation at the site of tumor
growth, can provide a quicker and more effective
treatment. Local administration, or inoculation into an
artery that directly supplies the area of tumor growth,
35 is advantageous because there is minimal dilution effect
and therefore a smaller dose is required to achieve

expression in a majority of the targeted cells. Other
modes of administration can be used as well. Such modes
are known and one skilled in the art will know which type
to use to treat a particular type of cancer.

5   Specific examples of treating cancer through
p53-mediated apoptosis using the above described gene
therapy methods include, for example, subjects with
breast or colon cancer metastasis to the liver. Such
subjects can have the vector delivered to tumors through
10   an hepatic artery infusion via a pump and indwelling
catheter. Bladder tumors can be treated via infusion of
vector into the bladder during cystoscopy and regional
lymph nodes can be treated through injection into
regional lymphatic vessels, for example. Other types of
15   tumor can be treated as well. Moreover, an additional
advantage of the described gene therapy methods is that
they can be used in conjunction with traditional cancer
therapies to further enhance eradication of the disease.

          The invention provides a method of selectively
20   reducing the viability of chronic granulocytic leukemia
cells within a sample of proliferating bone marrow cells.
The method includes (a) selectively arresting the
proliferation of normal cells within the sample of
proliferating bone marrow cells to generate a sample of
25   bone marrow cells containing arrested normal cells and
proliferating chronic granulocytic leukemia cells, and
(b) inducing p53-mediated apoptosis in the proliferating
chronic granulocytic leukemia cells.

          Chronic myeloid leukemia (CML) is a slowly
30   progressive disease that eventually results in death. One
form of treating this disease is to purge bone marrow
samples (autologous bone marrow) ex vivo of tumor cells
before reinfusion into a subject in an autologous bone
marrow transplant. The techniques currently employed for

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bone marrow purging, unfortunately, are time consuming
and not entirely efficient. Furthermore, they result in
some loss of stem cells. Clinical methods for preparing
bone marrow or peripheral blood hematopoietic cells for
5 autologous bone marrow transplantation are known in the
art. (Deisseroth, A.B. et al., _Human Gene Therapy_, 2:359
(1991)).

Depending on whether the chronic granulocytic
leukemia cells (CML) exhibit p53 activity and/or p53
10 suppressor activity, any of the above described methods
can be use to effectively kill CML cancer cells within
the population of bone marrow cells. One requirement to
selectively reduce the viability of only CML cells
however, is that the proliferation of the normal cells be
15 arrested. Hematopoietic stem cells within the population
are quiescent in G0 are not susceptible to p53-mediated
apoptosis.

Biologic response modifiers, such as growth
factors and cytokines are one means to achieve this
20 specificity. A specific example of a biologic response
modifier that can be use to specifically arrest normal
hematopoietic cells is macrophage inflammatory factor
(MIP-1α). This factor specifically arrests normal cells
in the G0 phase of the cell cycle. Thus, a population of
25 bone marrow cells can be treated with an amount of MIP-1α
that arrests normal cells and then p53-mediated apoptosis
can be induced in the susceptible proliferating CML
cells. Effective amounts of biologic response modifiers
are known within the art such as those described for MIP-
30 1α (Graham et al., _Nature_ 344: 442-444 (1990); Lord et
al., _Blood_ 79: 2605-2609 (1992)), or can be determined by
one skilled in the art by titrating the modifier and
determining at which concentration growth of normal cells
is inhibited.

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p53-mediated apoptosis can be induced using the
viral vector described previously, for example, to
express either a p53 encoding nucleic acid or antisense
to one or more p53-interacting regulatory proteins or
5   both. Alternatively, CML cells exhibiting, or made to
exhibit, p53 activity can be treated with antisense
oligonucleotides or analogues. Oligonucleotide analogues
provide an additional advantage because they can be made
to penetrate cellular membranes without the need for
10   specific transfection procedures. This advantage applies
to either _in vivo_ or _ex vivo_ treatment, or to _in vitro_
cultures. A specific example of an membrane permeable
oligonucleotide analog is phosphothioate
oligodeoxynucleotides.

15   It is understood that modifications which do
not substantially affect the activity of the various
embodiments of this invention are also included within
the definition of the invention provided herein.

The following examples are intended to
20   illustrate but not limit the present invention.

EXAMPLE I
Plasmid Construction

p53^{ts}neo was constructed from plasmid pLTRp53cG
(Eliyahu et al., _Nature (London)_ 316:158 (1985)). A Bam
25   HI site found in p53 intron sequences of pLTRp53cG was
destroyed by partial digestion with Bam HI, and blunt
ending with the large fragment of E. coli DNA polymerase
I in a buffer containing all four deoxynucleotides. The
plasmid was then recircularized with T4 DNA ligase. A
30   2.7 kb Nde I/Bam HI fragment from pSV-2neo containing the
neomycin resistance gene, was inserted into the remaining
Bam HI site of pLTRp53cG using Bam HI linkers. The 5'
Bam HI site was subsequently destroyed; p53^{ts}neo contains

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the coding sequence of the neomycin resistance gene inserted in the same orientation as the p53 coding sequences.

EXAMPLE II
p53-Mediated Apoptosis

Given the lack of expression of wild-type p53 in many MEL cell lines, to study the effect of wild-type p53 expression on MEL cell differentiation, an expression plasmid containing a minigene encoding a temperature sensitive p53, p53^{ts}neo (Ryan et al., supra), was electroporated into a p53-negative MEL cell line DP16-1, a MEL cell line which has no endogenous expression of p53 protein (Munroe et al., Mol. Cell. Biol. 10:3307 (1990); Mowat et al., Nature 314:633 (1985)). This mutant p53 contains a single base-pair mutation (alanine ---> valine at position 135) and can cooperate with ras in transforming primary rat embryo fibroblasts at 37.5°C. At 37.5°C the inactive ts-mutant p53^{ts} is predominantly cytoplasmic and is associated with the cellular heat shock protein hsc70. Clones expressing the mutant p53 grow rapidly at 37.5°C, however transfer to 32.5°C leads to growth arrest and subsequent loss of viability. In cells grown at 32.5°C, p53^{ts} becomes located in the nucleus (Ginsburg et al., Mol. Cell. Biol. 11:582 (1991)) and this mutant behaves as wild-type p53 as shown by its inhibition of growth and oncogene-mediated transformation (Michalovitz et al., Cell 62:671 (1990); Michalovitz et al., Mol. Cell. Biol. 11:582 (1991)).

DP16-1 MEL cells were grown in Dulbecco modified minimal essential medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of amphotericin B per ml. DNA transfections were performed by electroporation (Prochownik et al., supra). Transfection mixes consisted

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of 1 x 10⁷ cells in 400 μl transfection buffer (272 mM
sucrose, 7 mM phosphate (pH 7.4), 1 mM MgCl₂) with either
10 μg p53ᵗˢneo, or 10 μg pSV-2neo, linearized with Bam HI.
Transfected cells were selected in media containing 1

5   mg/ml geneticin (G418; GIBCO). Single-cell clones were
isolated by limiting cell dilution in 96-well microtiter
plates.

        To characterize the consequences of enforced
expression of p53 on differentiation, logarithmically
10 growing transfectants were exposed to media containing
1.6% dimethyl sulfoxide (DMSO) and incubated at 37.5°C.
Media was changed every other day. Cells underwent
growth arrest and subsequently died over a period of 48-
96 hours (Figure 8A; DP16-1 cells, ___, p53ᵗˢneo
15 transfected DP16-1 cells, _ _ _, and DP16-1 cells co-
transfected with c-myb and p53ᵗˢneo, _._._). Viable cells
were isolated by centrifugation through Ficoll-paque
(Pharmacia). Furthermore, benzidine staining for heme
expression (Gusella et al., Cell 9:221 (1976)) revealed
20 that only a small percentage of the p53ᵗˢneo transfected
cells had differentiated prior to cell death (Figure 8B;
legend as described for figure 8A). This is in contrast
to untransfected control DP16-1 cells which, when grown
in media containing DMSO, downregulate c-myb mRNA and
25 differentiate while remaining viable (Figure 8A & 8B).
As expected, the p53ᵗˢ protein was located in the
cytoplasm of the cells growing at 37.5°C in the absence
of DMSO (Figure 9A, panel a; C4 p53ᵗˢneo DP16-1 cells).
Surprisingly, even when grown at 37.5°C, exposure of the
30 p53ᵗˢneo transfected MEL cells to 1.6% DMSO led to the
relocation of the p53ᵗˢ protein in the nucleus (Figure 9A,
panel b; C4 cells) followed by cell death, showing that
DMSO can induce a mutant p53 to regain its function as a
tumor suppressor. Genomic DNA isolated from such cells
35 showed the oligonucleosomal "ladder" characteristic of
apoptosis (Figure 9B, lanes a & b; C4 and C9 p53ᵗˢneo

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transfectants, respectively). In addition, analysis with
conformation specific anti-p53 antibodies (Yewdell et
al., J. Virology 59:444 (1986)) showed the majority of
the p53^{ts} to be in the wild-type conformation. Thus
5   despite the fact that the cells were grown at 37.5°C,
DMSO caused the p53^{ts} to behave as wild-type p53 tumor
suppressor and induced apoptotic cell death.

EXAMPLE III
Nucleic Acid and Protein Analysis

10   Total cellular RNA was isolated from clones
growing at 37.5°C and purified by the guanidinium
thiocyanate/phenol extraction method (Chomczynski et al.,
Anal. Biochem. 162:156 (1987)). Northern blotting and
hybridization were performed as previously described
15   (Clarke et al., supra). To detect exogenous p53
expression the 3.5 kb Sma I fragment of p53^{ts}neo was used
as probe, labelled with [α-^{32}P] dCTP (Amersham) by random
priming using T7 DNA polymerase (Stratagene). Figure 1A
shows expression of p53 related message in the unselected
20   p53^{ts}neo transfected bulk culture (lane e), as well as in
three individual clones, C2, C4, and C9 (lanes b,c and d,
respectively), isolated from the bulk culture. A
transcript of approximately 2.1 kilobases is seen in the
transfected clones. This transcript is not seen in the
25   RNA from the parental cell line DP16-1 (lane a).

Genomic DNA was prepared as follows. Briefly,
cells were suspended in a buffer consisting of 20 mM
Tris, pH 7.5, 10 mM EDTA, 300 mM NaCl, lysed by adding
30   SDS to 0.5%, digested with proteinase K, followed by
organic extraction, and ethanol precipitation. DNA
samples (3 μg) were analyzed by electrophoresis through a
1.5% agarose gel containing 0.5 μg/ml ethidium bromide,
or through a non-denaturing 5% polyacrylamide gel.

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To confirm the expression of p53 protein in
transfected clones, cellular proteins were labelled by
incubating cells (1 x 10⁷) for 3 hours with 0.2 mCi of
[³⁵S]methionine in 1 ml of methionine-free DMEM. Cells
5 were lysed in lysis buffer (150 mM NaCl, 1% triton X-100,
50 mM Tris (pH 8.0), 0.5 mM phenylmethylsulfonyl
fluoride) and pre-cleared by overnight incubation at 4°C
with 50 μl of normal goat serum, followed by addition of
protein G sepharose beads (Pharmacia) and centrifugation.
10 The supernatant was immunoprecipitated with a mixture of
PAb421 (Harlow et al., J. Virology 39:861 (1981)), a pan-
specific monoclonal antibody against p53, and PAb248, a
murine-specific monoclonal antibody against p53. Immune
complexes were collected with protein A agarose beads
15 (BRL), washed 3x in lysis buffer and suspended in 25 μl
of Laemmli sample buffer. Samples were electrophoresed
through 10% polyacrylamide in the presence of SDS and
dried gels utilized for autoradiography. The p53 protein
is clearly seen in the transfected clone C9, but not in
20 parental line DP16-1 or in immunoprecipitations with
control monoclonal antibody (Figure 1B; clone C9
immunoprecipitated with p53 antibody (lane a), parental
line DP16-1 immunoprecipitated with p53 antibody (lane b)
and control monoclonal antibody (lane c), clone C9
25 immunoprecipitated with control monoclonal antibody (lane
d). Molecular weights (kd) are shown.).

To prepare cells for immunohistochemical
staining approximately 2.5 x 10⁵ cells were suspended in
buffer (phosphate buffered saline (PBS) -Ca⁺⁺, -Mg⁺⁺, 1%
30 bovine serum albumin fraction V, 0.5% tween-20, 5 μM
EDTA) and pelleted on a microscope slide using a cytospin
centrifuge. Cells were fixed with methanol for 60
minutes at 4°C and then washed twice with PBS. 40 μl of
a mixture of PAb421 and PAb248 was added to each slide
35 and allowed to stand for 60 minutes at room temperature.
Following a PBS wash, 25 μl of goat anti-mouse-FITC

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(Tago) was added for 60 minutes at room temperature.
After a final PBS wash the cells were counterstained with
Evans blue (Sigma) and viewed through fluorescence
microscopy. Immunohistochemistry shows that the p53
5   protein is located in the cytoplasm (arrowheads) of the
transfected cells, but not the parental cells, grown at
37.5°C, and is translocated to the nucleus when cells are
incubated at 32.5°C (Figure 1C; DP16-1 cells (left) and
DP16-1 cells transfected with p53^{ts}neo grown at 37.5°C
10 (middle) and 32.5°C (right)).

EXAMPLE IV
Cell Cycle Analysis Of p53 Induced Cell Death

The level of p53 protein varies in a cell-cycle
dependent manner in MEL cells (Khochbin et al., Exp. Cell
15 Res. 179:565 (1988)). Low levels are seen early in G1,
with a subsequent increase at the G1/S transition and a
more moderate increase occurring during the remainder of
S phase. A constant level is seen during G2/M, with a
return to low levels following division.

20   The relationship of p53 induced cell death to
the cell cycle was studied in transfected clone C9.
Clone C9 cells were synchronized in G0/G1 by density
arrest (typically at a concentration of 1-2 x 10^6
cells/ml) for 48-72 hours at 37.5°C. Cells were
25 stimulated to begin cycling by replating into fresh media
at a 1:10 split ratio. Growth arrest with mimosine
(Sigma) was accomplished by the addition of 300 μM
mimosine to logarithmically growing cells for a period of
24 hours.

30   Isoleucine deficient media was prepared using a
DMEM select-Amine Kit (Gibco). Logarithmically growing
cells were arrested by transfer to isoleucine deficient
media for a period of 18-24 hours.

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Following release from density arrest by
splitting 1:10 in fresh media, the cells were placed at
37.5°C and 32.5°C. At 37.5°C the cells progressed into S
and G2/M after a delay of approximately 20 hours (Figure

5   4A). Briefly, cell cycle analysis performed at the
following time-points after release from density arrest
showed: 0 hours (top left) 85% G1, 11% S, and 4% G2/M; 6
hours (top right) 75% G1, 23% S, and 2% G2/M; 20 hours
(bottom left) 55% G1, 44% S, and 1% G2/M; and 26 hours

10   (bottom right) 50% G1, 44% S, and 6% G2/M. Cells
released from density arrest at 32.5°C fail to begin
cycling secondary to a very rapid decrease in viability
(Figure 4B). The figure shows the cell cycle analysis
done 6 hours (top left), 9 hours (top right), and 12

15   hours (bottom) after release from density arrest.
Indicated are the DNA contents of avian erythrocytes
(line) and MEL cells in the G1 (1), S (2), and G2/M (3)
phases of the cell cycle. By 12 hours after release, the
majority of cells remain in G0/G1 though the sharpness of

20   the G0/G1 peak is lost as cell viability decreases.

Comparison of the kinetics of cell death with
that of a randomly growing population of clone C9 cells
placed at 32.5°C indicates that cells released into G1
following density arrest undergo cell death at an

25   accelerated rate (Figure 5; time at 37.5°C was: 0
hours,   6 hours,   9 hours,   15 hours,
28 hours). Almost complete cell death occurs by 15 hours
at 32.5°C, as opposed to 36-48 hours for a randomly
growing population. This indicated that cells released

30   into G1 may be preferentially susceptible to wild-type
p53 induced cell death. All six p53^{ts}neo clones examined
including, clones C2, C4, and C9, as well as the uncloned
G418 resistant bulk culture, showed similar kinetics
following transfer to 32.5°C (Figure 2; parental line

35   DP16-1.   Clone C4.   Clone C9). Six pSV-2neo
transfected clones, as well as the parental cell line

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DP16-1, showed no loss of viability when grown at 32.5°C.

To explore the possibility that cells released
into G1 may be preferentially susceptible to wild-type
p53 induced death, media was added to density arrested
5   cells and the cells were incubated for various times at
37.5°C, to allow progression out of G0. They were then
placed at 32.5°C and viability monitored. Cells released
from density arrest at 37.5°C for at least 12-15 hours
prior to being placed at 32.5°C began to show a survival
10 advantage over cells released directly at 32.5°C. This
advantage was more pronounced for cells allowed to
progress for 24-48 hours at 37.5°C (Figure 5). Analysis
of DNA content by quantitative flow cytometry of C9 cells
released from density arrest at 37.5°C for 36 hours prior
15 to placement at 32.5°C is shown in Figure 6. Briefly,
the cell cycle analysis of cells grown at 32.5°C for the
following times was: 0 hours (top left) 36% G1, 61.5% S,
and 2.5% G2/M; 6 hours (top right) 47% G1, 33% S, and 20%
G2/M; 15 hours (bottom left) 66.5% G1, 9% S, 24.5% G2/M;
20 and 20 hours (bottom right) 81% G1, 8% S, and 11% G2/M.
The DNA content of avian erythrocytes (vertical line) and
MEL cells in the G1 (1), S (2), and G2/M (3) phases of
the cell cycle are also shown.

What these results indicate is that at the time
25 the cells were downshifted to 32.5°C (0 hours), the
majority of cells have progressed into S phase. However,
after 6 hours at 32.5°C the percentage of S phase cells
has fallen significantly. This is attributable to a
progression of some cells into G2/M, while at the same
30 time the percentage of cells found in G1 also rises,
indicating a failure of cells to progress from G1 to S.
By 15 hours a majority of cells have accumulated in G1.
It is at this point that cell viability begins to
decrease (Figure 5). Cycling cells, therefore, progress

through S phase and G2/M and accumulate in G1 prior to
cell death occurring.

5   To rule out that G1 arrest alone induces cell
death, clones C9, C2 and C1 were growth arrested with
either mimosine (300 μM) (Watson et al., Cytometry 12:242
(1991)) or isoleucine deprivation (Heintz et al., Proc.
Natl. Acad. Sci. USA 79:4083 (1982)) at 37.5°C, both of
which result in late G1 arrest. These G1 arrested cells
do not undergo rapid cell death, though viability
10   decreases to approximately 70% after 3-4 days of arrest.
Viability falls to less than 5% within 36 hours in cells
arrested in G1 with either mimosine treatment or
isoleucine-deprivation and subsequent culture at 32.5°C,
indicating that cell death is specifically induced by
15   wild-type p53 expression.

       Cell cycle analysis was performed by fixing
cells (1-2 x 10^6 cells in PBS) with methanol, collecting
by centrifugation, and resuspending the cells in
propidium iodide (Sigma) stain (propidium iodide 50
20   μg/ml, 0.05% triton X-100, EDTA 18μg/ml, RNase A 100
units/ml, in phosphate buffered saline (PBS)). Avian
erythrocytes (1 x 10^6) were utilized as an internal
standard. After a 30 minute incubation at room
temperature DNA content was determined by quantitative
25   flow cytometry (Morasca et al., In (1986)).

EXAMPLE V
GO and Differentiated Cells are not Subject to p53
-Induced Cell Death

       To determine if release into G1 was a necessary
30   event for p53 to induce apoptosis, non-cycling GO cells
were placed at 32.5°C while still density arrested.
Simultaneously, density arrested cells were kept at
37.5°C and cells released into G1 (split 1:10) were
placed at both 37.5°C and 32.5°C. Viability was then

monitored. While cells released into G1 at 37.5°C grew
well and maintained a high viability, cells released at
32.5°C had the expected rapid loss of viability. At
37.5°C, density arrested p53^{t}neo cells will lose
5   viability slowly over time, and cell death is not
accelerated at 32.5°C. The same cells split 1:10 undergo
a much more rapid decline.

MEL cells can also exit the cell cycle by being
stimulated to terminally differentiate. DMSO was used to
10   induce differentiation of p53^{t}neo clones at 37.5°C.
After 5 days exposure to 1.6% DMSO, viable cells were
isolated by centrifugation through Ficoll-paque. The
cells obtained were terminally differentiated as shown by
1) high benzidine positivity (70-80%) and 2) failure to
15   proliferate when placed at 37.5°C in DMSO-free media.
These cells were placed at 32.5°C and viability
monitored. Such cells maintained viability over several
days, while control randomly growing cultures underwent
the expected cell death.

20   EXAMPLE VI
         Commitment to Cell Death is Cell Cycle Dependent

         To delineate the time course of commitment to
cell death, density arrested C9 cells were split 1:10 in
fresh media and placed for varying periods of time at
25   32.5°C. They were then returned to incubation at 37.5°C.
Viability was checked 18 hours after the cells had been
released from density arrest. Cells which spent less
than 3 hours at 32.5°C maintained a high viability (Table
1). Between 3 and 4 hours, however, the majority of
30   cells became committed to cell death, as shown by a low
viability at 18 hours. By 6 hours at 32.5°C
approximately 90% of the cells are committed to
undergoing cell death. Cells which remained viable at 18
hours were able to proliferate if kept at 37.5°C,

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indicating that they had escaped commitment to cell
death.

TABLE I
Time course of commitment to p53 induced cell death

5　　　　　　Density arrested clone C9 cells were split 1:10
and placed at 32.5°C for varying periods of time before
transfer to 37.5°C.　Viability was then determined 18
hours after release from density arrest.

Time at 32.5°C (hours)	Viability at 18 hours
1	83%
2	79%
3	67%
4	30%
6	12%

15　Density arrested clone C9 cells were split 1:10, allowed
to cycle for 36 hours at 37.5°C, then placed at 32.5°C
for varying periods of time, prior to transfer to 37.5°C.
Viability was determined 18 hours after placement at
32.5°C.

Time at 32.5°C (hours)	Viability at 18 hours
4	>90%
6	>90%
8	>90%
10	>90%
18	68%

In hematopoietic cells, p53 mRNA expression peaks in S phase (Khochbin et al., supra). Clone C9 cells which, following release from density arrest, were allowed to cycle at 37.5°C for 36 hours into S phase
5   (Figure 6) do not show an early commitment to cell death when placed at 32.5°C (Table 1). Although some cells have lost viability after 18 hours at 32.5°C, if returned to 37.5°C many of the surviving cells will continue to proliferate. In contrast to cells in G1, where a brief
10   exposure to p53 commits cells to die, even a prolonged exposure to wild-type p53 during S phase and G2/M does not appear to lead to an irreversible commitment to cell death.

EXAMPLE VII
15   DNA Fragmentation Occurs with Commitment to Cell Death

In order to understand the molecular events which lead to p53 induced MEL cell death, cells were analyzed for the appearance of DNA fragmentation. Cells were released from density arrest at 32.5°C, and genomic
20   DNA was isolated at various time points.

Commitment to cell death correlates with the initial appearance of low molecular weight DNA fragments (Figure 7). The lanes are labelled in figure 7 with the number of hours cells were cultured at 32.5°C prior to
25   DNA isolation. Also shown is Hind III-digested lambda phage DNA and Hae III-digested PhiX174 phage DNA used as a molecular weight marker (M). This occurs after 4.5-6 hours at 32.5°C (though cell viability remains greater than 90% at this time) in density arrested cells released
30   into G1. Although appearing to be normal when viewed by light microscopy, the majority of these cells will lose viability despite being returned to 37.5°C.

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EXAMPLE VIII
Integration of p53 and c-myb in DP16-1 Cells

To determine how c-myb and p53 interact during
MEL cell growth and differentiation, DP16-1 cells were
5   co-transfected with p53^{ts}neo and pMbml-dhfr, which encodes
a full-length c-myb cDNA under the control of an SV40
promoter (Clarke, et al., supra). Whereas DP16-1 cells
co-transfected with a control dhfr plasmid and p53^{ts}neo
underwent apoptosis (Fig. 9B), MEL cells co-transfected
10 with pMbml-dhfr and p53^{ts}neo did not undergo rapid cell
death when exposed to 1.6% DMSO at 37.5°C (Figure 9A;
panel c, C5a p53^{ts}neo/c-myb transfected DP16-1 cells;
panel d, C5a cells). Instead, these cells proliferated
for 2-3 days, and then arrested coincident with an
15 increase in benzidine-positive cells (Figure 11).
Viability decreased only after the majority of cells had
differentiated.

Increased expression of c-myb was selected for
by growing transfected cells in higher concentrations of
20 methotrexate (Clarke et al., supra). Whereas DP16-1 MEL
cells transfected with pMbml-dhfr alone were highly
resistant to DMSO-induced differentiation, amplified
cultures of p53^{ts}neo/pMbml-dhfr co-transfectants showed a
significant degree of differentiation. These cultures
25 also did not proliferate indefinitely, as do the
pMbml-dhfr transfectants, but instead lost viability
after 10-14 days in 1.6% DMSO.

To further characterize the interactions of
these genes, DP16-1 clones C5a and C31a, which expressed
30 p53 (Figure 10A; C5a (lane a), C31a (lane b), or DP16-1
(lane c)) and had significantly amplified c-myb mRNA
levels , were isolated by limiting dilution. Figure 10B
shows c-myb RNA levels as determined by RNase mapping.
C5 and C31 are clones grown in 0.25 µM methotrexate, and

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C5a and C31a are the respective clones grown in 4 µM
methotrexate. The arrow shows the exogenous c-myb band.
Figure 10C shows the amplification of c-myb genomic DNA
sequences (C5 (0.25 µM), C5a (4 µM), C31 (0.25 µM), and

5   C31a (4 µM) cells) whereas figure 10D shows c-myb RNA
levels as determined by northern blot (DP16-1 cells (lane
a), 84B cells (lane b), C5a cells (lane c), and C31a
cells (lane d)). To detect exogenous p53 expression of
mRNA from clones C5a and C31a, the 3.5 kb Sma I fragment

10   of p53ᵗˢneo was used as probe, labeled with [α-³²P] dCTP
(Amersham) by random priming using T7 DNA polymerase
(Stratagene).

     RNase mapping of c-myb was accomplished using
total RNA (10 µg) incubated with 1 x 10⁵ cpm of ³²P

15   labeled riboprobe for quantification of exogenous c-myb
mRNA (Zinn et al., Cell 34:865 (1983)). The riboprobe
vector was made by sub-cloning the Bam HI-Eco RI fragment
of pMbml (Weber et al., supra) into pGEM 1. The
resultant plasmid was digested with Pvu II, and a

20   riboprobe generated using SP6 polymerase. The riboprobe
and indicated mRNA was incubated at 50°C for 16 hours,
digested with RNase A and T1, and analyzed on a
denaturing acrylamide gel (Zinn et al., supra). The
undigested riboprobe is approximately 340 bases, and the

25   Mbml specific message is 140 bases.

     Amplification of the transfected c-myb DNAs was
analyzed using genomic DNA that was isolated from C5 and
C31 grown in 0.25 µM methotrexate, and C5a and C31a grown
in 4 µM methotrexate was isolated by lysing cells in SDS,

30   followed by proteinase K digestion, organic extraction
and ethanol precipitation.

     A 35 base oligodeoxynucleotide complimentary to
bases 3271-3305 of human c-myb was labeled with T4 kinase

and gamma ^{32}P ATP and used to probe a Northern blot to
detect expression of human c-myb.

In contrast to cells transfected with p53^{ts}neo
and dhfr, which rapidly die, C5 and C31 displayed
5   intermediate levels of viability when exposed to media
with DMSO (Figures 11A; p53^{ts}neo transfected DP16-1 cells,
____, C5 cells, _ _ _, and C5a cells, _._._, & 11C;
p53^{ts}neo transfected DP16-1 cells, ____, C31 cells, _ _ _,
and C31a cells, _._._). After further amplification of
10 c-myb , the clones designated C5a and C31a, continued to
grow when cultured in 1.6% DMSO at 37.5°C (Figures 11A &
11C). Although there was some loss of viability, these
clones continued to proliferate indefinitely despite the
DMSO-induced translocation of p53 into the nucleus
15 (Figure 9, panel c & d). These data show that
constitutive expression of high levels of c-myb mRNA
overcomes the anti-proliferative effect of p53.

EXAMPLE IX
20   p53 Inhibition of c-myb

Since c-myb is able to partially protect the
cells from p53-induced apoptosis, it was next determined
whether p53 has any effects on the c-myb differentiation
block. In contrast to a control pMbm1/dhfr-transfected
25 DP16-1 MEL cell culture, which exhibited the expected
high level expression of c-myb and resultant inhibition
of differentiation, DP16-1 cells co-transfected with
c-myb and p53 partially differentiated when grown in 1.6%
DMSO. This implies that the presence of intranuclear p53
30 influences cells to differentiate despite the
constitutive expression of c-myb. The extent of MEL cell
differentiation is inversely related to the level of
c-myb mRNA (Clarke et al., supra). Before amplification
of c-myb, a high percentage of C5 and a moderate
35 percentage of C31 cells could differentiate in response

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to DMSO (Figure 11B; 84P cells, ____, C5 cells, _ _ _,
and C5a cells, _._._, and 11D; 84B cells, ____, C31
cells, _ _ _, and C31a cells, _._._). In C31 cells, this
lower level of differentiation was probably secondary to
5   a more modest protection from apoptosis (Figure 11C).
After c-myb amplification, C5a and C31a cells still
showed substantial levels of induced differentiation
(Figures 11B & 11D), although the time course was delayed
in comparison to the control DP16-1 cells (Figure 8B).

10   In contrast, c-myb transfected DP16-1 cells and
84B cells, a MEL cell line constitutively expressing a
similar level of c-myb mRNA as C5a and C31a (Figure 11D),
remained benzidine negative despite prolonged culture in
1.6% DMSO. As clone C5a and C31a cells were passaged in
15   DMSO-containing media a significant percentage remained
benzidine positive for several weeks. This indicates
that new cells continued to become committed to terminal
differentiation while the population as a whole retained
a proliferative capacity. To confirm that these results
20   were not unique to the DP16-1 MEL cell line, 84B MEL
cells were transfected with p53. A clone was isolated,
shown to express p53 and the exogenous c-myb, and induced
to differentiate with DMSO. These cells behaved in a
manner identical to C5a and C31a cells, i.e., a
25   significant number of cells differentiated despite
constitutive expression of c-myb. Thus, c-myb appears to
delay differentiation in these cells, but, presumably
because of p53 expression, does not block it completely.

EXAMPLE X

30   Coimmunoprecipitation of p53 and c-myb

To determine whether the effects of co-
expression of c-myb and p53 might be due to a direct
interaction between the two proteins, it was determined

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whether antibodies to c-myb would coimmunoprecipitate
p53.

C5a cells were seeded at a density of 4 x 10⁵
cells/ml and incubated at 32.5°C for fourteen hours.
5   Proteins were labeled as follows: 3 x 10⁶ cells were
washed three times in serum and methionine free media,
and incubated for three hours in 7 ml of methionine free
DMEM, 10% dialyzed calf serum, and 150 μCi of ³⁵S
methionine. The cells were collected by centrifugation
10 and lysed in 400 μl of RIPA buffer (10 mM Tris, pH 7.6,
1 mM EDTA, 150 mM NaCl, and 0.5 mM PMSF) with 0.2% triton
X-100. The extracts were clarified by centrifugation at
12/,000 x g for 10 minutes and used for co-
immunoprecipitations.

15   Briefly, 5 μl of the first antiserum was added
to 100 μl of sample, and samples were rocked at 4°C for 4
hours followed by the additions of 30 μl of Staph G
sepharose (Pharmacia) and incubated for an additional
hour. The beads were pelleted in a microfuge for 10
20 seconds and washed 5 times in 800 μl of RIPA buffer with
triton X-100. Protein complexes were disrupted by adding
400 μl of RIPA buffer with 0.1% SDS and 0.5%
deoxycholate. The beads were pelleted, and the
supernatants were transferred to new eppendorf tubes,
25 centrifuged, and the supernatant again transferred to a
new eppendorf tube. Twenty μl of anti-p53 monoclonal
antibody (pAb 421) was then added, and the samples
incubated overnight. Protein G sepharose beads were
added, and samples incubated for an additional hour. The
30 beads were then collected by centrifugation, and washed
three times with RIPA buffer. After collecting the
beads, they were resuspended in 25 μl of SDS sample
buffer, and the bound proteins analyzed by SDS-PAGE and
autoradiography.

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Shown in figure 12 are the following
immunoprecipitations: lane a, 1st antibody was the pre-
immune serum from the rabbit used to raise the anti-c-myb
antibodies. Lane b, 1st antibody was serum from the
5   rabbits immunized with recombinant bacterial anti-c-myb.
Lane c, 1st antibody was sheep anti-c-myb. Lane d, 1st
antibody was nonspecific goat serum. These results show
that both c-myb and anti-sera, but not preimmune serum or
non-specific serum, coimmunoprecipitated p53.
10   Furthermore, the p53 protein was not coimmunoprecipitated
with c-myb in 84B cells which do not express p53, or if a
nonspecific antibody was used in place of the anti-p53
antibody for the second immunoprecipitation.

EXAMPLE XI
15   p53 downregulates c-myc mRNA levels

To determine the effects p53 has upon c-myc
mRNA expression, cultures of p53^{ts}neo-transfected DP16-1
cells which had been density arrested for two days were
released from density arrest either at 32.5°C or 37.5°C.
20   RNA was extracted at times zero, two, four, and six hours
after release from density arrest. In two separate
experiments, expression of the wild-type, but not mutant,
p53 resulted in the downregulation of c-myc mRNA (Figure
13).

25   DP16-1 cells were density arrested for two
days. Cells, typically at a density of 1-2 x 10^6
cells/ml, were then released from density arrest by
diluting cells to a concentration of 4 x 10^5 cells/ml with
fresh medium. The cells were incubated at the indicated
30   temperature for the indicated period of time. Cells were
then harvested, and RNA was isolated by the method of
Guanidine isothiocyanate lysis and CsTFA centrifugation.
Twenty micrograms of RNA was electrophoresed through an
agarose/formaldehyde gel and blotted onto a nylon

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membrane. Northern blots were probed with a mouse c-myc
cDNA Xho I fragment labeled with α^{32}P dCTP using random
primers essentially as previously described. The 2.4 kb
c-myc message is indicated. Note that cells grown at
5 32.5°C rapidly loss expression of c-myc mRNA.

This experiment eliminates one possible
mechanism by which p53 induces apoptosis in cells. It
has been previously reported that expression of c-myc in
cell cycle arrested cells results in apoptosis. Thus,
10 since p53 is known to block cells in G1, it is possible
that the p53-induced G1 cell block killed MEL cells
because c-myc continued to be expressed. Instead, these
data imply that p53 might exert at least some of its
effects through downregulation of c-myc expression. This
15 observation is consistent with our earlier finding that
p53 accelerated death of MEL cells blocked in G1 by
agents other than p53. These data support a model in
which p53 acts as a failsafe mechanism for cell cycle
regulation.

20   EXAMPLE XII
                C-myc blocks p53-induced apoptosis

To determine whether c-myc modulates p53-
induced effects, DP16-1 cells were transfected with
p53^{ts}neo and pXVmyc/dhfr and selected cells by sequential
25 growth in G418 and methotrexate. As a negative control,
DP16-1 cells were cotransfected with p53^{ts}neo and
pSVLZquad/dhfr (a vector in which the 4 leucines in the
myc leucine zipper have been mutated). Total cellular
RNA was isolated from the bulk culture and individual
30 clones, and was analyzed by RT/PCR for expression of the
transfected and mRNA (Figure 14a) and by Northern blot
for expression of p53 (Figure 14b). The expression of
p53 protein in these cells was confirmed by
immunoprecipitation with antibodies directed to p53.

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For Figure 14a, one μg of RNA was transcribed
into cDNA using MuLV RT (Superscript, BRL). One
twentieth of the cDNA was amplified with TAQ DNA
polymerase and a 3′ primer homologous to the c-myc cDNA
5   and a 5′ primer homologous to transcribed SV40 sequences
unique to the exogenous myc mRNA. The expected 310 bp
fragment was present in the transfected clones, but not
in the DP16-1 controls. Shown in figure 14c is twenty
micrograms of RNA were analyzed by Northern blot   The
10   probe was the 3.5 kb p53 Sma fragment from p53^{ts}neo. As
expected, all the transfected clones, but not the
parental cell line, express p53.

EXAMPLE XIII
Conformation of p53^{ts} in DMSO-treated DP16-1 Cells

15   DMSO induces the ts mutant p53 to assume the
wild-type phenotype, translocate to the nucleus, and
causes cell death. Protein extracts from the DMSO-
treated cells were probed with conformation specific
antibodies, the majority of the p53^{ts} assumed the wild-
20   type phenotype (Figure 15). p53^{ts}neo co-transfected MEL
cells were grown in medium containing 1.6% DMSO for 24
hours. Cells were then washed and resuspended in
methionine-free medium containing 1.6% DMSO and ^{35}S
methionine for three hours. Cells were harvested, and
25   lysed in 10 mM Tris pH 7.5, 75 mM KCl, 1 mM EDTA, 0.25 mM
PMSF, and 0.1% triton X-100. After removal of cellular
debris, the protein extract was divided into equal
aliquots and immunoprecipitated with p53 monoclonal
antibody pAb421 (which recognizes both the wild-type and
30   mutant conformation), pAb246 (which recognizes only the
wild-type phenotype), and pAb240 (which recognizes only
the mutant phenotype). Note that the majority of the
newly synthesized p53 is in the wild-type conformation.

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EXAMPLE XIV
Viability and Differentiation of DP16-1 Cells Transformed with p53 and c-myc

Amplified c-myc/p53^{ts}neo transfected bulk
5   culture and several individual clones, as well as control
pLZquad/dhfr/p53^{ts}neo transfected cells, were incubated in
medium containing 1.6% DMSO. As expected, DP16-1 cells
maintained viability and differentiated, and cells co-
transfected with pLZquad/dhfr and p53 died. In contrast,
10   cells transfected with both p53 and c-myc maintained
viability (Figure 16), but differentiated. Symbols
presented in the figure are as follows: parental line
DP16-1: p53^{ts}neo transfected DP16-1 cell bulk culture;
, p53^{ts}neo and pSVmyc/dhfr cotransfected DP16-1 cell
15   bulk culture;   , p53^{ts}neo and pSVmyc/dhfr cotransfected
DP16-1 cells;   , p53^{ts}neo and pSVmyc/dhfr cotransfected
DP16-1 cell bulk culture (amplified myc); and   ,
p53^{ts}neo and pSVmycLZquad/dhfr (mutant myc) cotransfected
DP16-1 cells.

20   Thus, cells transfected with myc, but not a
mutant c-myc, behave in an analogous manner as cells
transfected with c-myb and p53. At 0 hours DMSO was
added to a final concentration of 1.6% to the medium of
growing cells.   Differentiation was monitored by
25   benzidine staining. Note that the parental cell line
maintained a high viability until cells became terminally
differentiated, p53^{ts}neo of pSVmyc/dhfr cells rapidly
died, and cells transfected with both plasmids has
intermediate viability and differentiation patterns.
30   DP16-1 cells cotransfected with pSV2neo or pSV2neo and
pFR400 (dhfr) behaved in a manner identical to the
parental cell line.

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EXAMPLE XV
Enhancement of MEL cell differentiation by c-myc
in the absence of mutant p53

MEL cell lines invariability contain mutations
5   of the p53 tumor suppressor gene and express either
negligible or mutant p53 protein. During MEL
differentiation there is a biphasic decline in expression
of the c-myc oncogene although enforced expression of c-
myc blocks this differentiation. To determine the
10 relationship between mutant p53 and c-myc in cell
differentiation, DP16-1 cells (which have deleted both
p53 alleles) were transfected with an amplifiable plasmid
vector containing a full-length mouse c-myc cDNA with or
without plasmid vector containing a mutant p53 cDNA
15 (p53^{pro193}). The exogenous c-myc cDNA is transcribed in MEL
cells and results in increased expression of c-myc mRNA
and protein during DMSO-induced differentiation.

Extent of differentiation was monitored by
benzidine staining (Figures 17A and 17B). When MEL cells
20 are exposed to DMSO, differentiation normally occurs over
a five day period. In contrast to the parental DP16-1
cells or DP16-1 cells transfected with pSV$_2$LZquad, c-myc
transfected DP16-1 cells or underwent accelerated
differentiation. The parental cells remained largely
25 undifferentiated after two days of exposure to medium
containing DMSO, while about 70-80% of the c-myc
transfected cells were differentiated. Furthermore, c-
myc transfected DP16-1 cells stopped growing and
eventually lost viability when grown in medium containing
30 1.6% DMSO. Coincident expression of p53^{pro193} with c-myc
abolished the accelerated DMSO-induced differentiation
seen in cells which expressed c-myc alone.

These results show that rather than blocking
differentiation, enforced expression of c-myc in the

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absence of p53 accelerates differentiation. The
accelerated differentiation is reversed by coincident
expression of mutant p53 with c-myc. Thus, enforced
expression of c-myc can potentiate a differentiation
5   signal and mutant p53 can overcome this potentiation.

                          EXAMPLE XVI
                 BCL2 delays p53-induced apoptosis

         MEL cells were transfected with p53^{ts} hygro
     (constructed by replacing the p53^{ts}neo neomycin resistance
10   gene with a hygromycin resistance gene) or p53^{ts} hygro and
     p^{ssv}bcl2neo. DP16-1 cells which express p53^{ts} or p53^{ts} and
     bcl2 were selected. When wild-type p53 was analyzed by
     growing transfected cells at 32.5°C, the cells expressing
     p53^{ts} rapidly die over a two day period. However, cells
15   coexpressing p53^{ts} and bcl2 maintain viability until day
     four.

                          EXAMPLE XVII
         Purging CML Cells from Low Density Mononuclear Bone
     Marrow Cell With Antisense myb

20   CML cells often are selectively depleted during
     culture, this depletion, however, is variable and slow.
     Patients with CML can be treated by autologous bone
     marrow transplantation using hematopoietic cells which
     have been cultured in the presence of c-myb antisense
25   oligodeoxynucleotides.

         The most successful purging of bone marrow
     cells includes from one-to-three non-overlapping
     techniques. CML cells display antigens which are not
     present on the hematopoietic stem cells. A large
30   majority of the CML cells that contaminant Marrow samples
     can initially be removed using antibodies that recognize
     these CML-specific antigens. Antibodies that can be used
     include specificities directed to one or more of the

following antigens: CD15, CD33, Glycophorin A, CD10, CD2, CD4 and CD8. Antibody-cell conjugates can be removed by either flow cytometry, immuno-magnetic beads or compliment, for example. The remaining CML cells can be
5 killed by incubating the enriched culture in the presence of c-myb antisense oligonucleotides.

Briefly, mononuclear cells will be isolated from a 5 ml bone marrow aspirate by centrifuging through a ficol-hypaque density gradient. The isolated bone
10 marrow cells ($1x10^5$ CD34+ progenitor cells) are then cultured in Dexter medium with IL3, SGF and EPO and macrophage inflammatory factor (MIP-1α; 300 μg/ml) for 24 hours and then are cultured in medium containing c-myb antisense oligonucleotides. MIP-1α arrests normal
15 hematopoietic cells in G0 and these cells will therefore be resistant to c-myb antisense. The CML cells, however, are unresponsive to MIP-1α and will continue to cycle. For c-myb antisense treatment, the oligonucleotide 5'-GTGCCGGGGTCTTCGGGC-3' is added at a concentration of 40
20 μg/ml and the culture is changed two times a day for a period of from 1-to-three days. The cells remaining viable in culture after this period will be only the normal hematopoietic cells.

The above treated cell culture is reinfused
25 into the donor patient. This procedure effectively purges a bone marrow sample of cancer cells, while maintaining the viability of normal cells.

Although the invention has been described with reference to the disclosed embodiments, those skilled in
30 the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the

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spirit of the invention. Accordingly, the invention is
limited only by the following claims.

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We claim:

     1.   A method of reducing the viability of a
proliferating mammalian cell deficient in p53 activity
and in p53 suppressor activity of one or more p53-
5 interacting regulatory proteins comprising increasing the
level or activity of p53 in said cell.

     2.   The method of claim 1, wherein said
proliferating mammalian cell is a cancer cell.

     3.   The method of claim 2, wherein said cancer
10 cell is a leukemic cell, lymphoma cell, breast cancer
cell, prostate cancer cell, oat cell carcinoma cell, lung
cancer cell, colon cancer cell, bladder cancer cell,
brain tumor cell or pancreatic cancer cell.

     4.   The method of claim 1, wherein said one or
15 more p53-interacting regulatory proteins is c-myb, c-myc
or bcl2.

     5.   The method of claim 1, wherein said level
or activity of p53 is increased by expressing a p53
encoding nucleic acid in said proliferating mammalian
20 cell.

     6.   A method of reducing the viability of a
proliferating mammalian cell exhibiting p53 activity and
p53 suppressor activity of one or more p53-interacting
regulatory proteins comprising reducing the suppressor
25 activity of said one or more p53-interacting regulatory
proteins.

     7.   The method of claim 6, wherein said
proliferating mammalian cell is a cancer cell.

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8.   The method of claim 7, wherein said cancer
cell is a leukemic cell, lymphoma cell, breast cancer
cell, prostate cancer cell, oat cell carcinoma cell, lung
cancer cell, colon cancer cell, bladder cancer cell,
5   brain tumor cell, head and neck cancer cell or pancreatic
cancer cell.

9.   The method of claim 6, wherein said one or
more p53-interacting regulatory proteins is c-myb, c-myc
or bcl2.

10   10.   The method of claim 6, wherein said
suppressor activity is reduced by decreasing the level of
said one or more p53-interacting regulatory proteins.

11.   The method of claim 10, wherein said level
is decreased by introducing into said cell a nucleic acid
15   substantially complementary in sequence to a nucleic
acid encoding said one or more p53-interacting regulatory
proteins.

12.   The method of claim 11, wherein said
nucleic acid substantially complementary in sequence to a
20   p53-interacting regulatory protein encoding nucleic acid
is an antisense oligonucleotide or analog.

13.   A method of reducing the viability of a
proliferating mammalian cell deficient in p53 activity
and exhibiting p53 suppressor activity of one or more
25   p53-interacting regulatory proteins, comprising:
            (a)   increasing the level or activity of p53 in
said cell, and
            (b)   reducing the suppressor activity of said
one or more p53-interacting regulatory proteins.

30   14.   The method of claim 13, wherein said
proliferating mammalian cell is a cancer cell.

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15. The method of claim 14, wherein said
cancer cell is a leukemic cell, lymphoma cell, breast
cancer cell, prostate cancer cell, oat cell carcinoma
cell, lung cancer cell, colon cancer cell, bladder cancer
5   cell, brain tumor cell, head and neck cancer cell or
pancreatic cancer cell.

16. The method of claim 13, wherein said level
or activity of p53 is increased by expressing a p53
encoding nucleic acid in said proliferating mammalian
10 cell.

17. The method of claim 13, wherein said one
or more p53-interacting regulatory proteins is c-myb,
c-myc or bcl2.

18. The method of claim 13, wherein said
15 suppressor activity is reduced by decreasing the level or
activity of said one or more p53-interacting regulatory
proteins.

19. The method of claim 18, wherein said
suppressor activity is reduced by introducing into said
20 cell a dominant-negative mutant of said one or more p53-
interacting regulatory proteins.

20. The method of claim 18, wherein said level
is decreased by introducing into said cell a nucleic acid
substantially complementary in sequence to a nucleic
25 acid encoding said one or more p53-interacting regulatory
proteins.

21. The method of claim 20, wherein said
nucleic acid substantially complementary in sequence to a
p53-interacting regulatory protein encoding nucleic acid
30 is an antisense oligonucleotide or analog.

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22. A method of selectively reducing the viability of proliferating cancer cells compared to nonproliferating normal cells within a mixed population of cells comprising selectively inducing p53-mediated
5 apoptosis in said proliferating cancer cells.

23. The method of claim 22, wherein said cancer cell is a leukemic cell, lymphoma cell, breast cancer cell, prostate cancer cell, oat cell carcinoma cell, lung cancer cell, colon cancer cell, bladder cancer
10 cell, brain tumor cell, head and neck cancer cell or pancreatic cancer cell.

24. The method of claim 22, wherein said p53-mediated apoptosis is selectively induced by increasing the level or activity of p53 in said proliferating cancer
15 cells.

25. The method of claim 24, wherein said level or activity of p53 is increased by expressing a p53 encoding nucleic acid.

26. The method of claim 22, wherein said p53-
20 mediated apoptosis is selectively induced by reducing the suppressor activity of one or more p53-interacting regulatory proteins.

27. The method of claim 26, wherein said suppressor activity is reduced by introducing into said
25 cell a dominant-negative mutant of said one or more p53-interacting regulatory proteins.

28. The method of claim 26, wherein said one or more p53-interacting regulatory proteins is c-myb, c-myc or bcl2.

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29.   The method of claim 26, wherein said
suppressor activity is reduced by decreasing the level of
said one or more p53-interacting regulatory proteins.

30.   The method of claim 29, wherein said level
5   is decreased by introducing into said cell a nucleic acid
substantially complementary in sequence to a nucleic
acid encoding said one or more p53-interacting regulatory
proteins.

31.   The method of claim 30, wherein said
10   nucleic acid substantially complementary in sequence to a
p53-interacting regulatory protein encoding nucleic acid
is an antisense oligonucleotide or analog.

32.   The method of claim 22, wherein said p53-
mediated apoptosis is selectively induced by increasing
15   the level or activity of p53 in said proliferating cancer
cells, and reducing the suppressor activity of said one
or more p53-interacting regulatory proteins.

33.   The method of claim 32, wherein said level
or activity of p53 is increased by expressing a p53
20   encoding nucleic acid.

34.   The method of claim 32, wherein said one
or more p53-interacting regulatory proteins is c-myb,
c-myc or bcl2.

35.   The method of claim 32, wherein said
25   suppressor activity is reduced by decreasing the level or
activity of said one or more p53-interacting regulatory
proteins.

36.   The method of claim 35, wherein said level
is decreased by introducing into said cell a nucleic acid
30   substantially complementary in sequence to a nucleic

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acid encoding said one or more p53-interacting regulatory
proteins.

37.   The method of claim 36, wherein said
nucleic acid substantially complementary in sequence to a
5   p53-interacting regulatory protein encoding nucleic acid
is an antisense oligonucleotide or analog.

38.   A method of selectively reducing the
viability of chronic granulocytic leukemia cells within a
sample of proliferating bone marrow cells, comprising:
10   (a)   selectively arresting the proliferation of
normal cells within said sample of proliferating bone
marrow cells to generate a sample of bone marrow cells
containing arrested normal cells and proliferating
chronic granulocytic leukemia cells, and
15   (b)   inducing p53-mediated apoptosis in said
proliferating chronic granulocytic leukemia cells.

39.   The method of claim 38, wherein said
proliferation of normal cells within said sample of
proliferating bone marrow cells is selectively arrested
20   by contacting said sample with an effective amount of a
biologic response modifier.

40.   The method of claim 39, wherein said
biologic response modifier is MIP-1α.

41.   The method of claim 38, wherein said p53-
25   mediated apoptosis is induced in said proliferative
chronic granulocytic leukemia cells by the method of
claim 1, 6 or 13.

42.   A method of inducing p53 tumor suppressor
activity in a cell having a mutant p53 gene product,
30   comprising treating the cell with an effective amount of

53

DMSO sufficient to induce said mutant p53 gene product to assure a wild-type conformation or activity.

43. The method of claim 42, wherein said mutant p53 gene product is p53^{ts}.
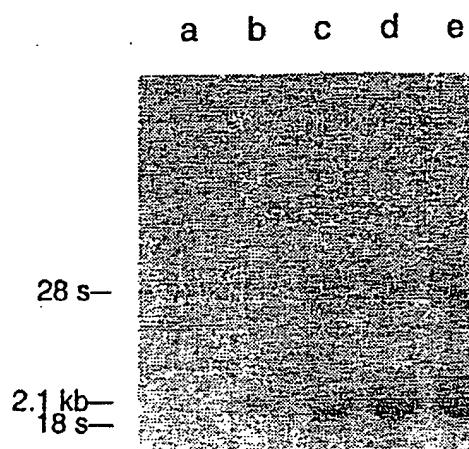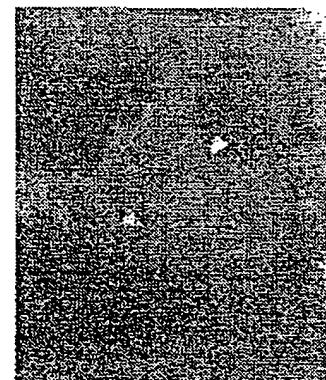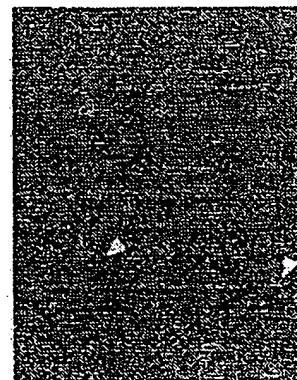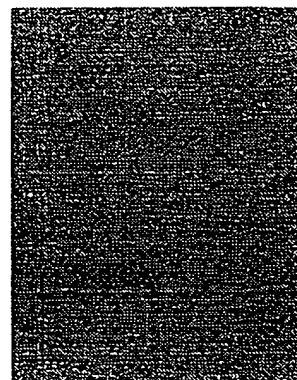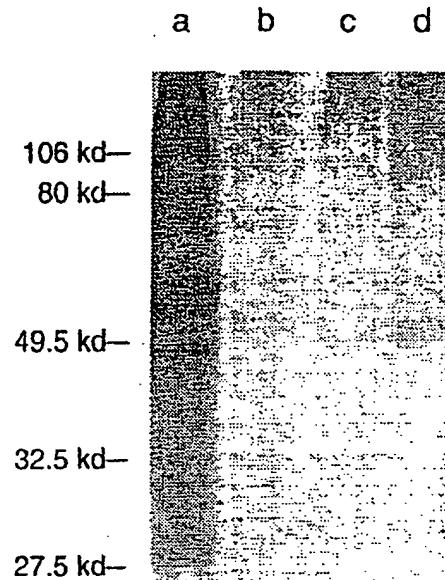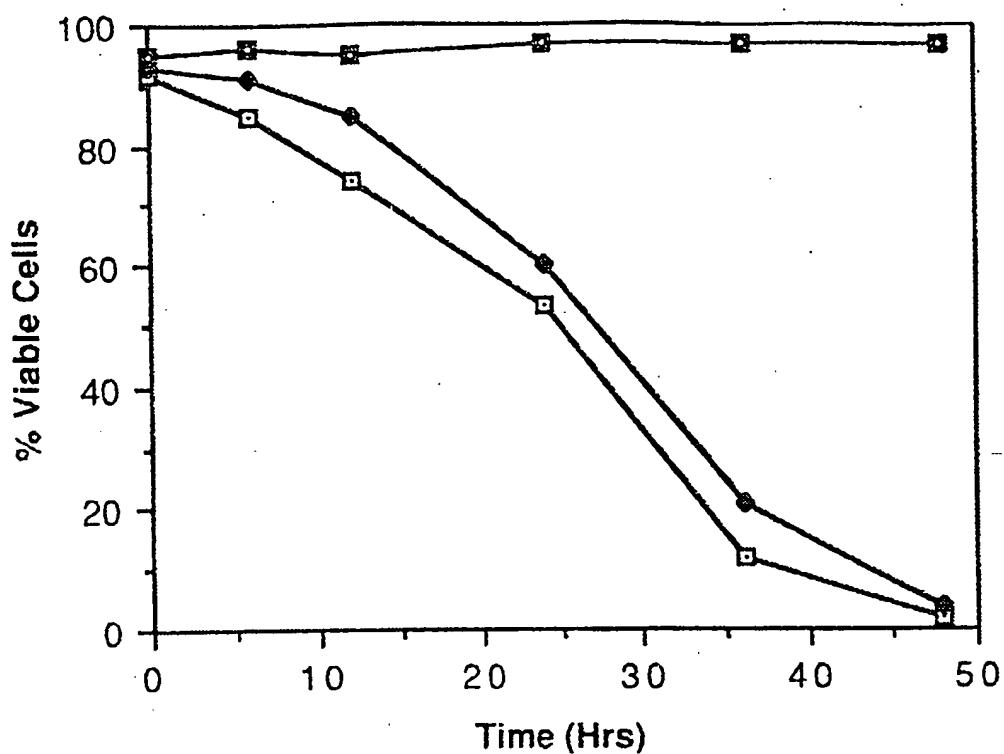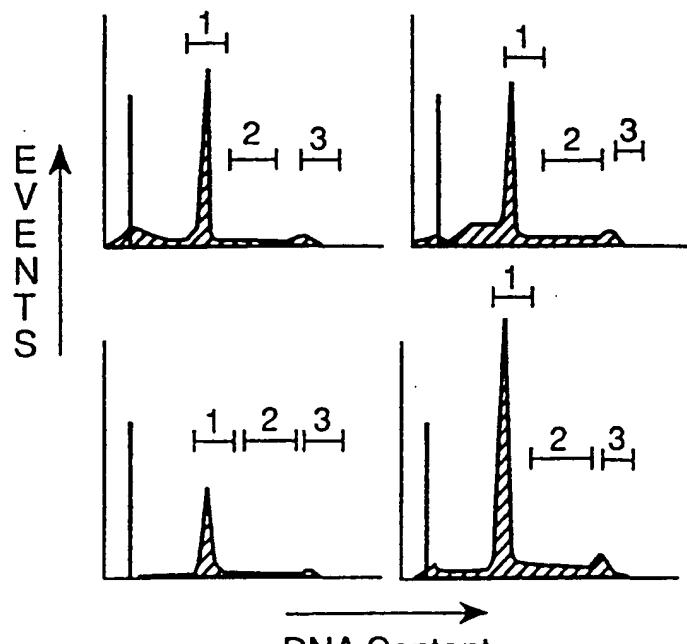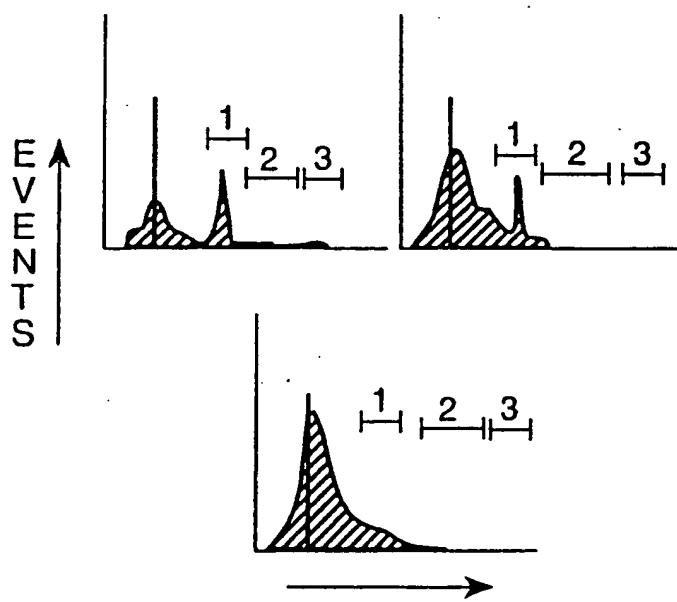
FIG.1A

a b c d e

28 s—

2.1 kb—
18 s—

FIG.1B

a b c d

106 kd—
80 kd—

49.5 kd—

32.5 kd—

27.5 kd—

FIG.1C

FIG.2

a b c

FIG.3

DNA Content

FIG.4A

DNA Content

FIG.4B

FIG.5

DNA Content
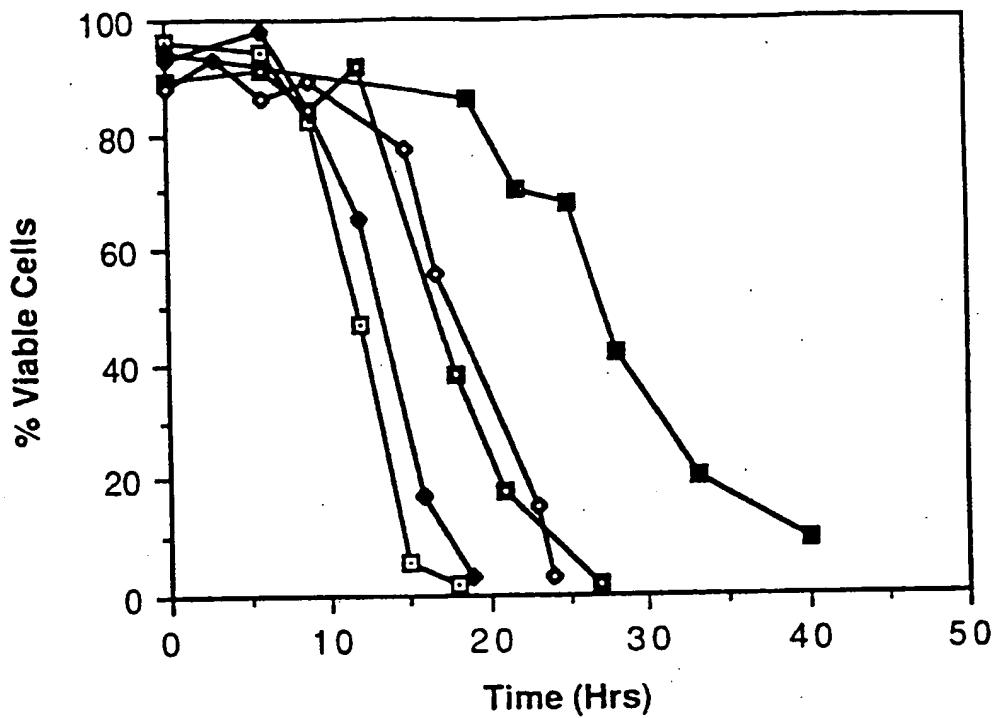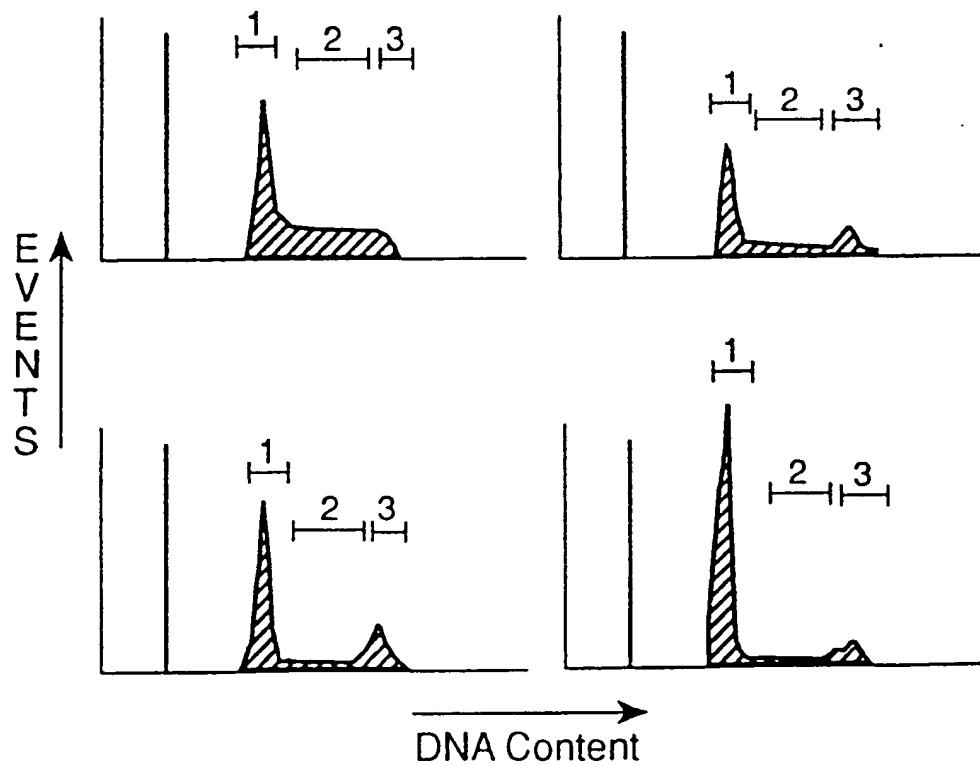
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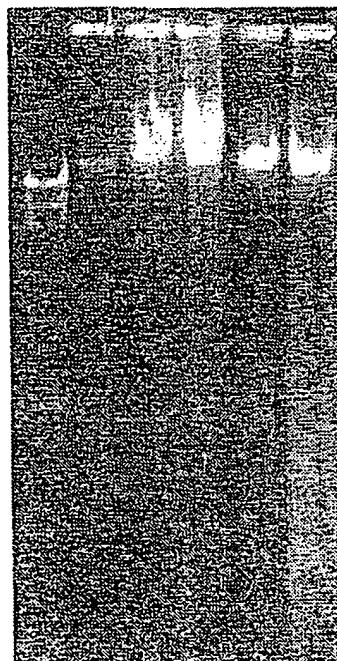
FIG.7
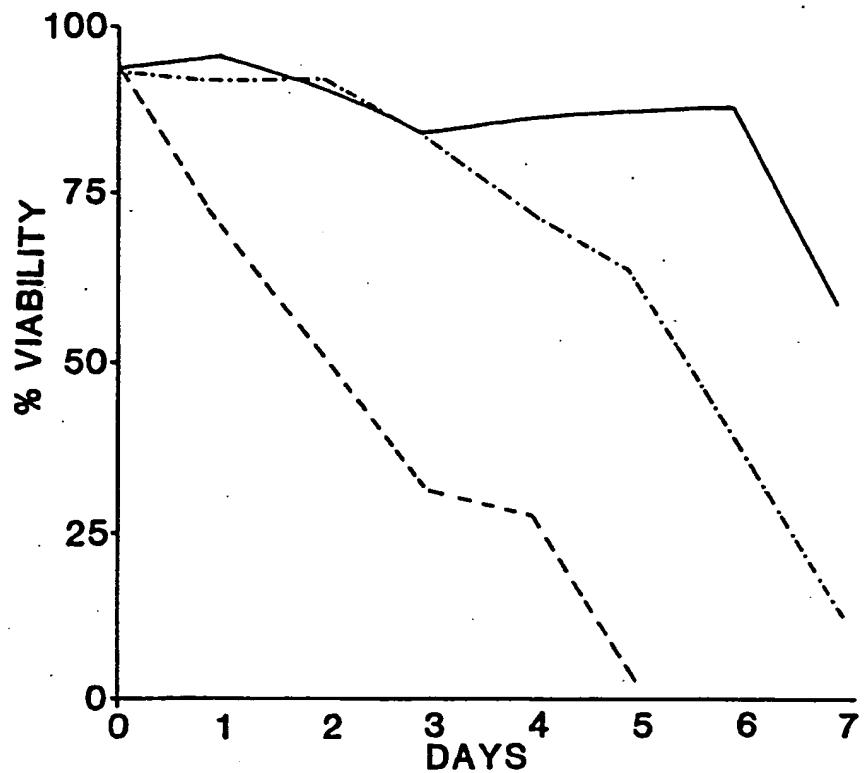
FIG.8A
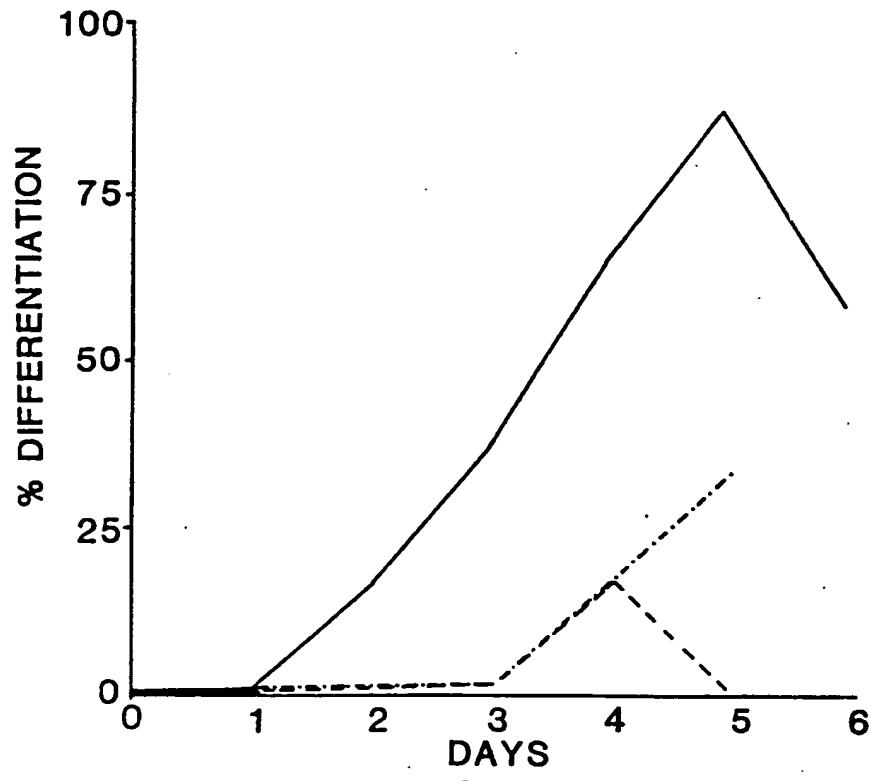
FIG. 8B

FIG.9A-1

FIG.9A-2

FIG.9A-3

FIG.9A-4

a   b   c
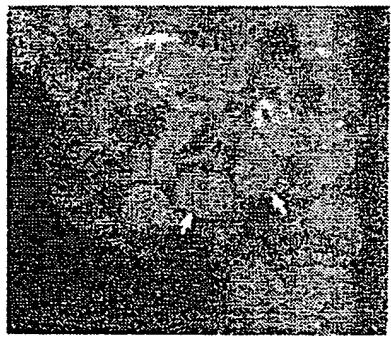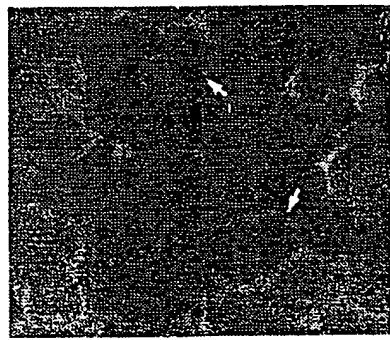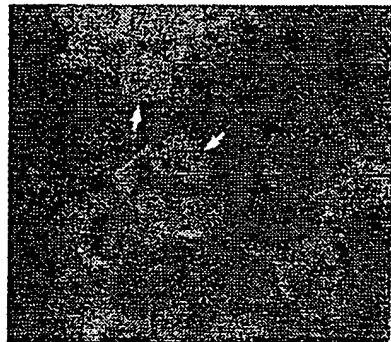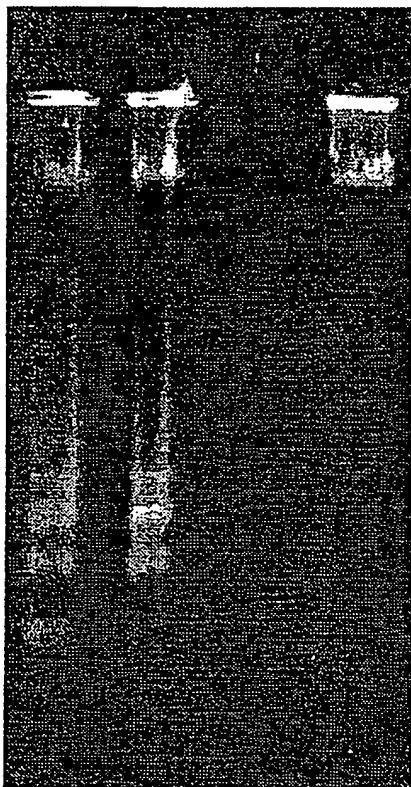
FIG. 9B
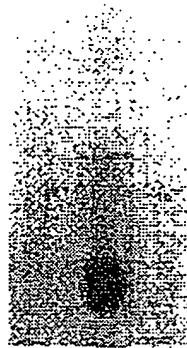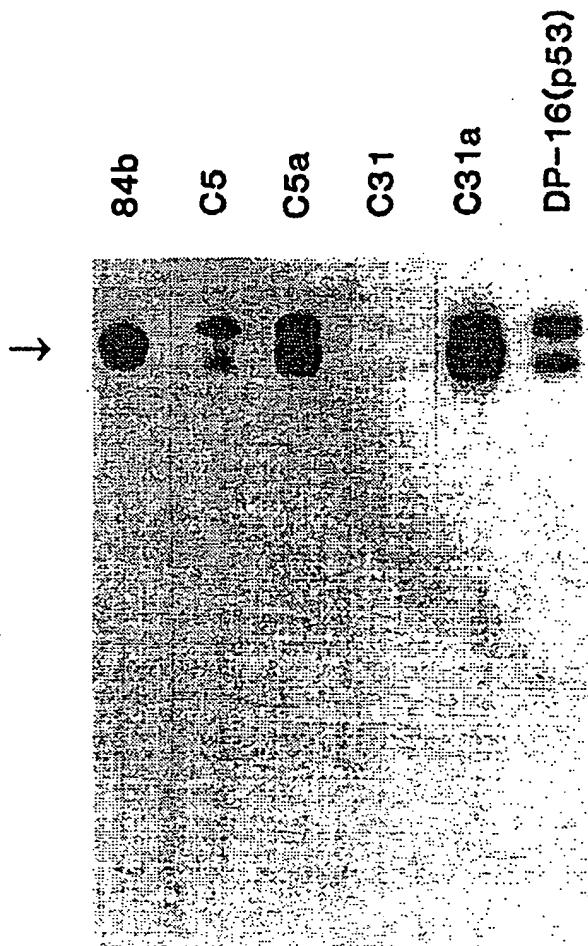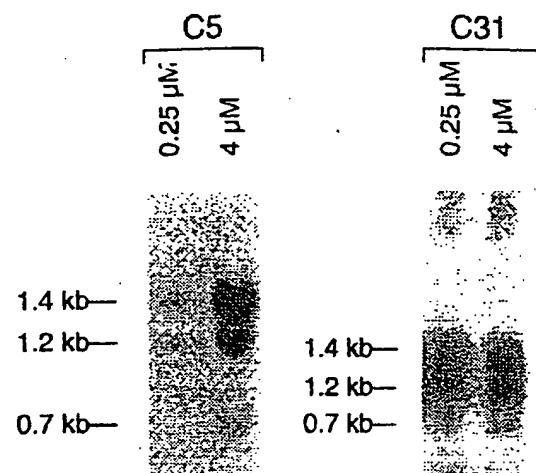
FIG.10A
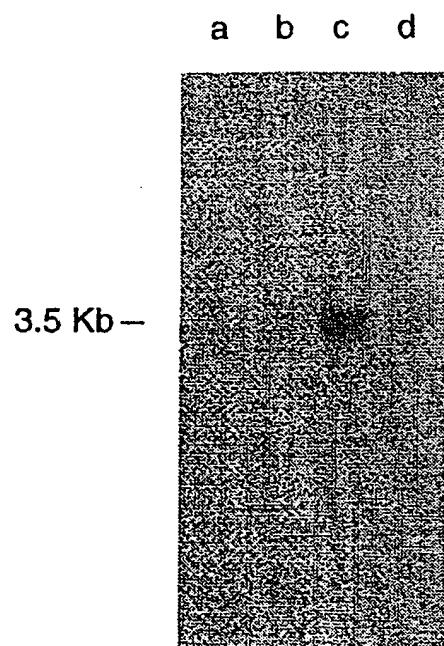
FIG.10B

FIG.10C

FIG. 10D

FIG.11A
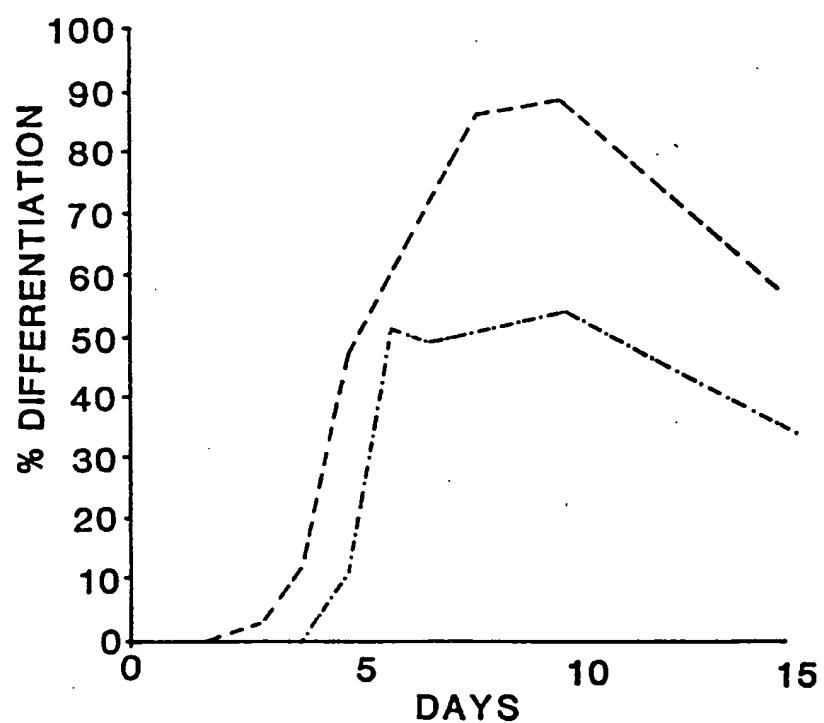
FIG.11B
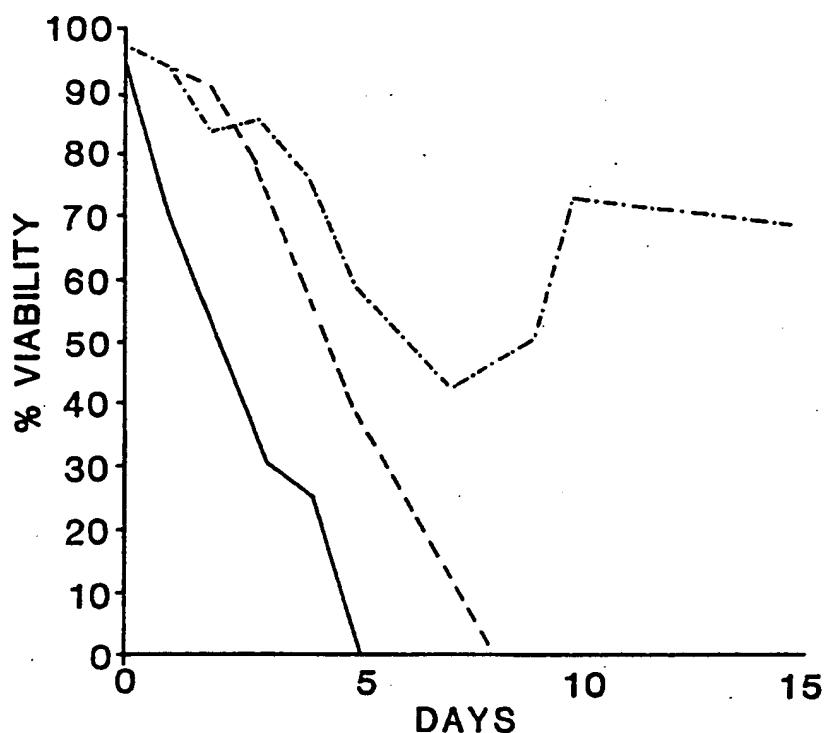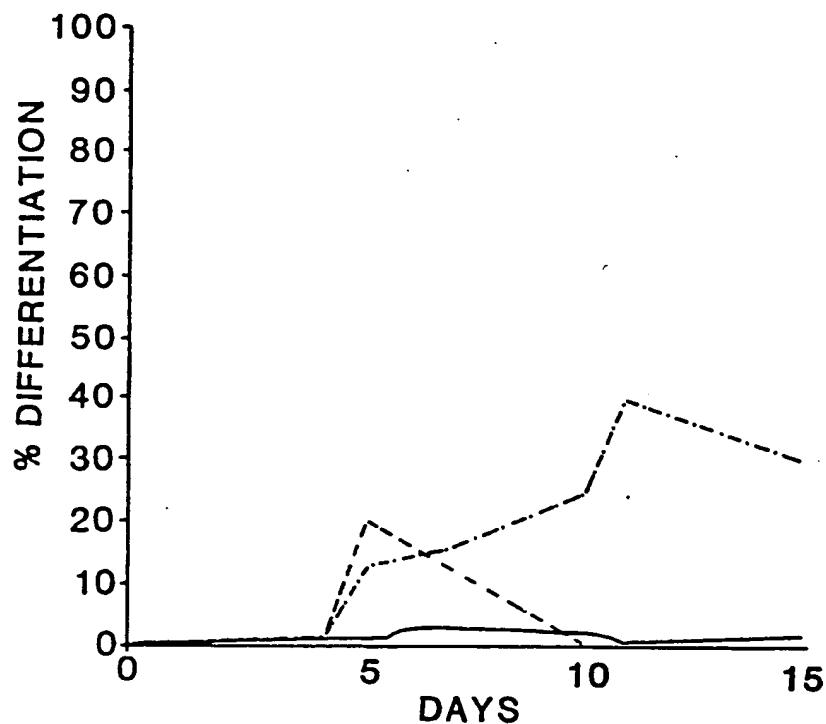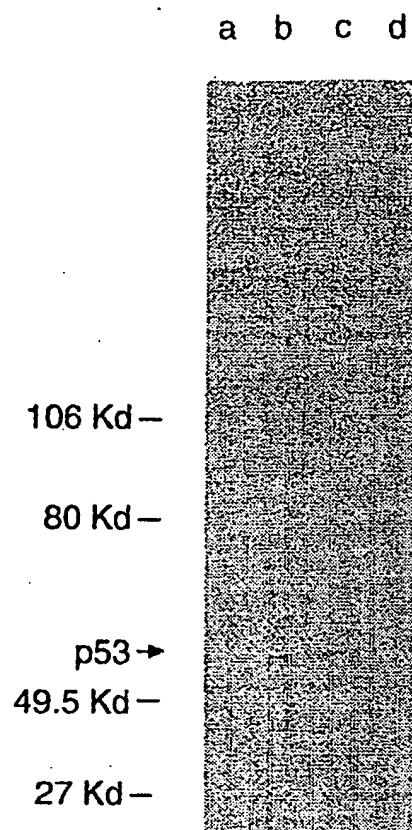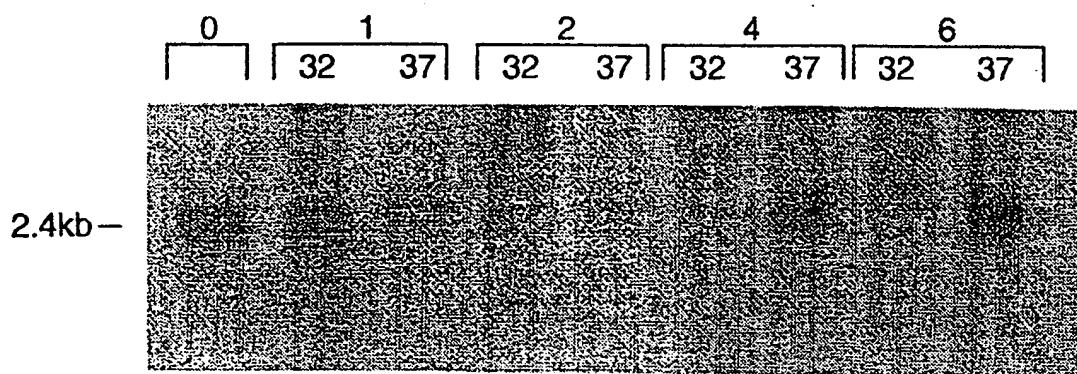
FIG.11C

FIG.11D

FIG.12

FIG.13

14 / 17

FIG.14a

FIG.14B

FIG. 15

TIME (HOURS)
FIG.16

VIABILITY

● p53myc 2a
□ p53myc 2
○ p53ts neo/dhfr
■ p53myc LZquad
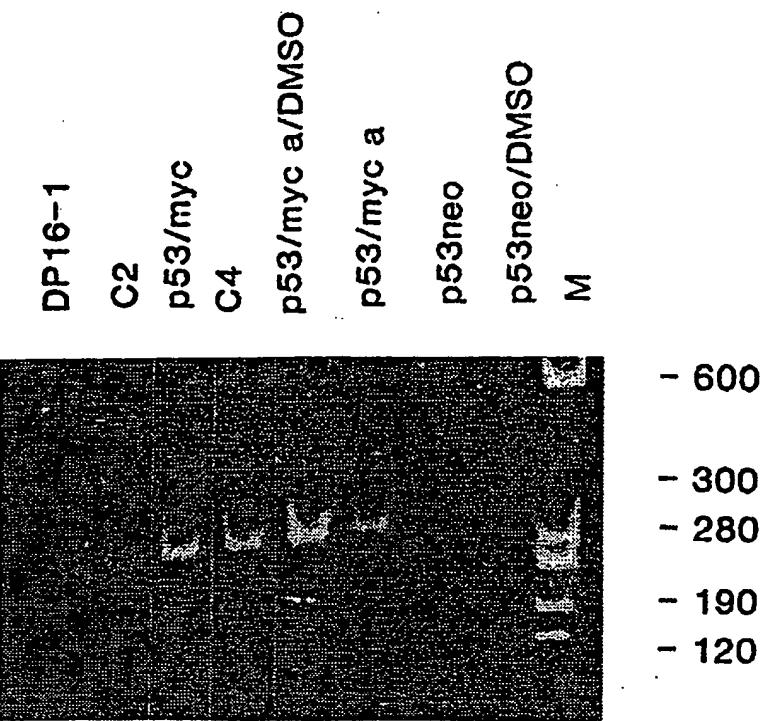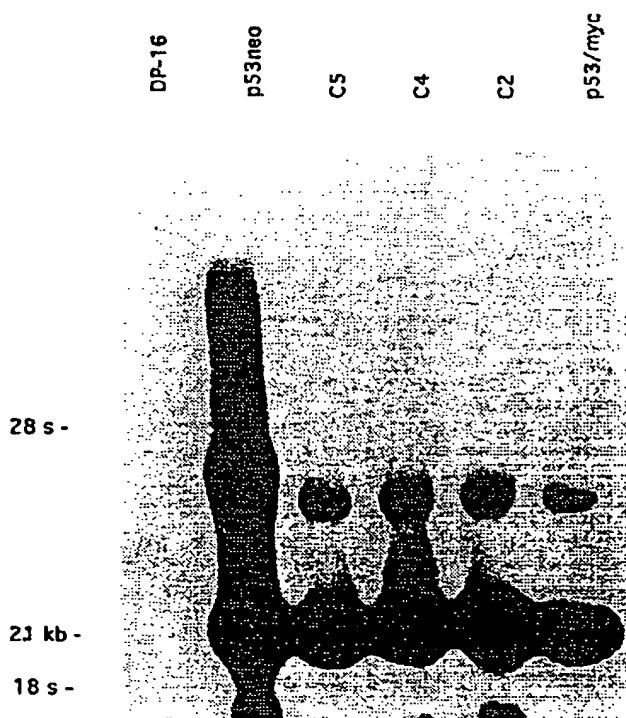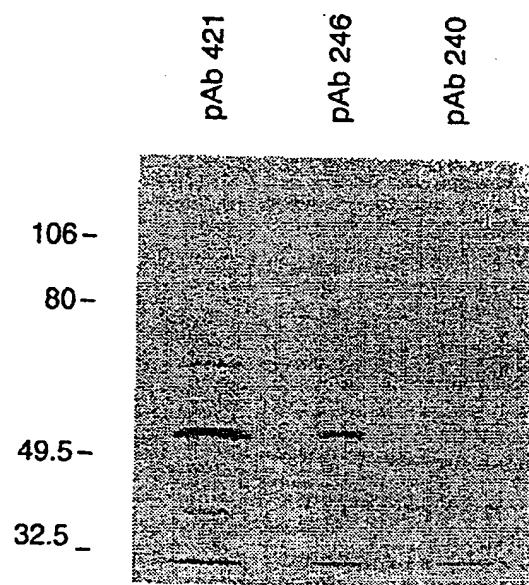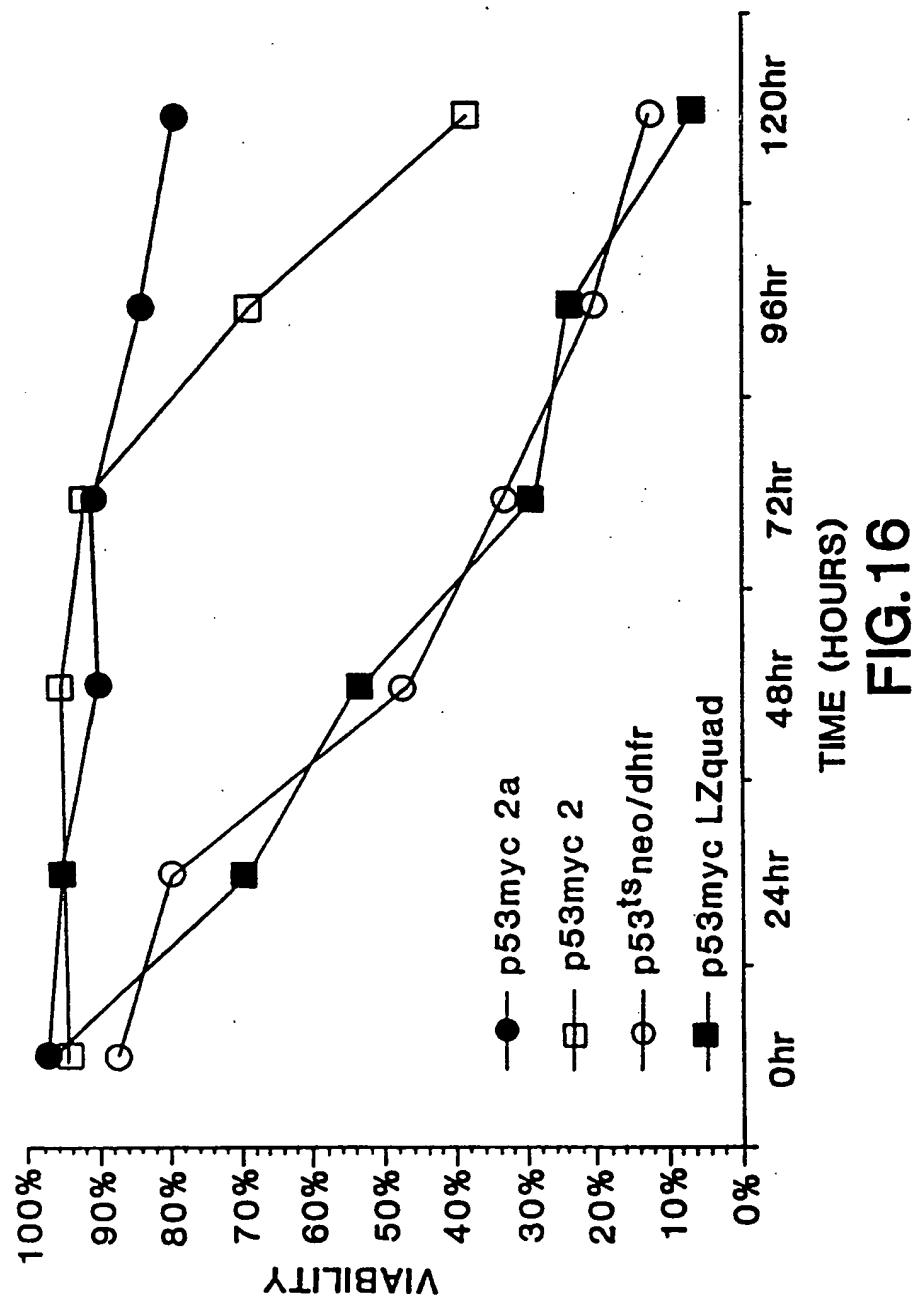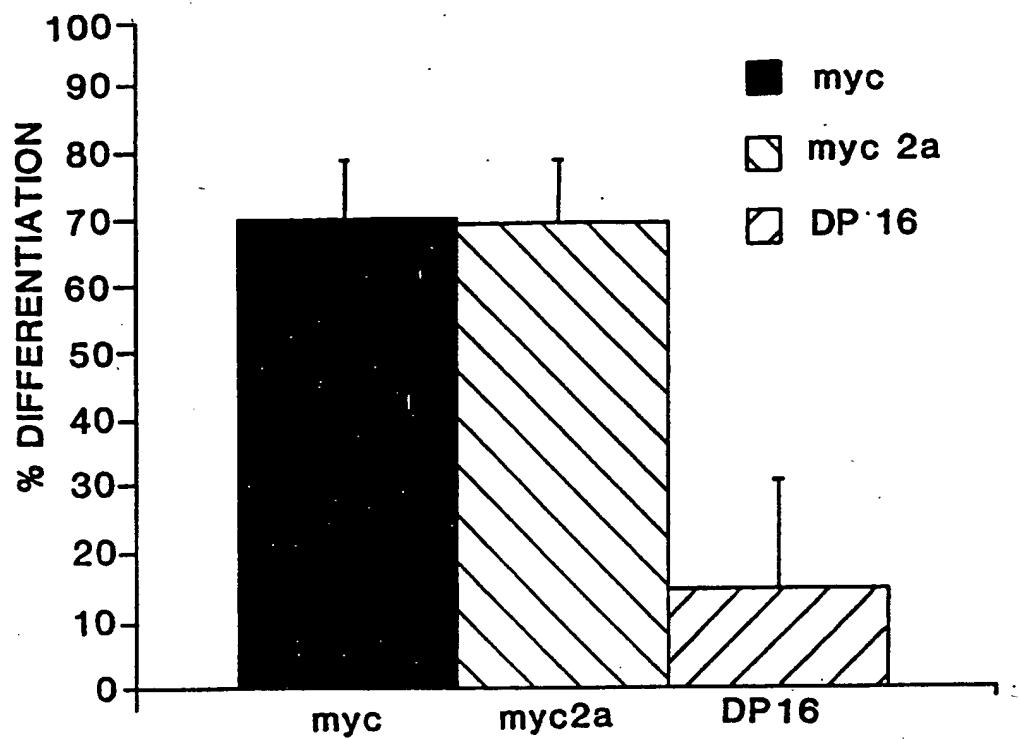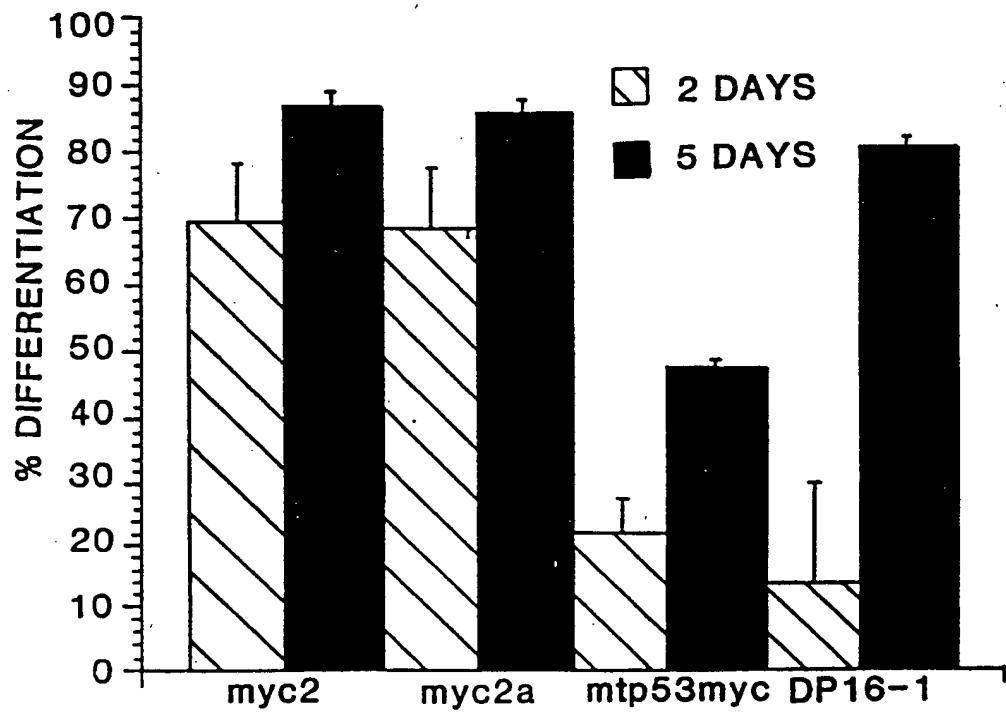
FIG.17A

FIG.17B

	Interna. Application No
	PCT/US 94/11923

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/12   C12N15/11   A61K48/00   //C07K14/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C07K   A61K   C12N

Documentation searched other than minimum documentat~ :o the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 15580 (RESEARCH DEVELOPMENT FOUNDATION) 17 October 1991	1-37
Y	see page 2, line 24 - page 5, line 15; claim 9; figure 6 ---	38-41
Y,0	LEUKEMIA AND LYMPHOMA, vol.11 SUPPL, 1993, SWITZERLAND pages 245 - 253 Eaves C et al 'The biology of normal and neoplastic stem cells in CML.' & Chronic myeloid leukemia, 2nd international conference,Bologna,Italy,October 4-7, 1992 see the whole document --- -/--	38-41

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 1995	2 0. 01. 95
Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INTERNATIONAL JOURNAL OF HEMATOLOLOGY, vol.57, no.3, June 1993, IRELAND pages 197 - 206 Lord BI et al 'Macrophage inflammatory protein: its characteristics, biological properties and role in the regulation of haemopoiesis.' see page 203, left column, paragraph 4 - page 204, right column, paragraph 1 ---	38-41
X	EP,A,0 475 623 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 18 March 1992 see page 8, line 5 - line 25 see page 10, line 35 - line 51 ---	1-5
X	WO,A,93 09789 (TEMPLE UNIVERSITY- OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) 27 May 1993 see claims 1-6; examples 3,4 ---	6-12,22, 26-31
X	WO,A,93 20200 (IMPERIAL CANCER RESEARCH TECHNOLOGY LTD.) 14 October 1993	6-12
A	see claims 1-8 ---	21
X	BIOLOGICAL ABSTRACTS, vol. 87 Philadelphia, PA, US; abstract no. 018048, KLINKEN S P ET AL 'TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF C-MYC C-MYB AND P53 DURING PROLIFERATION AND DIFFERENTIATION OF MURINE ERYTHROLEUKEMIA CELLS TREATED WITH DFMO AND DMSO' see abstract	42
Y	& EXP CELL RES, 178 (2). 1988. 185-198. ---	43
Y	BIOLOGICAL ABSTRACTS, vol. 95 Philadelphia, PA, US; abstract no. 106018, JOHNSON P ET AL 'GROWTH SUPPRESSION OF FRIEND VIRUS-TRANSFORMED ERYTHROLEUKEMIA CELLS BY P53 PROTEIN IS ACCOMPANIED BY HEMOGLOBIN PRODUCTION AND IS SENSITIVE TO ERYTHROPOIETIN' see abstract & MOL CELL BIOL, 13 (3). 1993. 1456-1463. -----	43

3

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 1-43, at least partially as far as they concern in vivo methods of treatment, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                             [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9115580	17-10-91	AU-A-	7750191	30-10-91
		CA-A-	2079903	11-10-91
		CN-A-	1056427	27-11-91
		EP-A-	0527804	24-02-93
EP-A-0475623	18-03-92	AU-B-	653356	29-09-94
		AU-A-	8262991	27-02-92
WO-A-9309789	27-05-93	AU-A-	3070992	15-06-93
WO-A-9320200	14-10-93	NONE		